

Determining biological factors associated with chemotherapy response in glioblastoma patients

Author:

Sevim, Hatice

Publication Date:

2012

DOI:

<https://doi.org/10.26190/unsworks/15784>

License:

<https://creativecommons.org/licenses/by-nc-nd/3.0/au/>

Link to license to see what you are allowed to do with this resource.

Downloaded from <http://hdl.handle.net/1959.4/52224> in <https://unsworks.unsw.edu.au> on 2023-02-18

Determining Biological Factors Associated with Chemotherapy Response in Glioblastoma Patients

By Hatice Sevim

Cure for Life Foundation Neuro-oncology Laboratory,
Lowy Cancer Research Centre, Prince of Wales Clinical
School, Faculty of Medicine,
University of New South Wales

A thesis submitted in partial fulfilment of the requirements for the
degree of Doctor of Philosophy (PhD)

February 2012

ORIGINALITY STATEMENT

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'



Signed :

Date: 12.11.2012

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: **SEVIM**

First name: **HATICE**

Other name/s:

Abbreviation for degree as given in the University calendar: **PhD**

School: **Prince of Wales Clinical School**

Faculty: **Faculty of Medicine**

Title: **Determining Biological Factors Associated with Chemotherapy**

Response in Glioblastoma Patients

ABSTRACT

Glioblastomas are characterised by remarkably high resistance to chemotherapy. The majority of patients are unresponsive to standard therapy including concurrent chemo/radiotherapy and adjuvant chemotherapy with TMZ. Investigation of potential contributors of chemoresistance is needed so that patients who do not respond can avoid futile chemotherapy and be better matched to something that might work.

The aim of this thesis was to investigate factors associated with chemotherapy resistance. The expression of single biomarkers were examined, namely Topoisomerase II alpha (TopoII α), Neuro/glia2 (NG2) and MGMT and their association with response to etoposide (TopoII α -inhibitor), cilengitide (α v β 3 and α v β 5 integrin-inhibitor) and temozolomide, respectively. We found that the response to therapy in each case was much more complex than one drug and one biomarker. Rather, resistance is defined by the interplay of multiple proteins and genes. In response, I took a global proteomic approach to discover novel biomarkers which might be associated with response and survival outcome of the patients. Data collected using chemically-modified ProteinChip surfaces revealed significantly differentially expressed peaks between non-responders vs. responders and STS vs. LTS. Biomarker panels including multiple proteins were generated. A panel of three proteins (combined predictability = 100%) overexpressed in non-responders were further identified as DEFA3, MIF and S100A8. MIF protein was up-regulated both in non-responders and STS. The expression of these biomarkers was also examined in a validation cohort (n = 66) using IHC which we are not yet able to extract entire data. All three proteins serve as components of pro-inflammatory and inflammatory tumour microenvironment and have important roles in pro-inflammatory and pro-tumorigenic processes. These features are not taken into consideration in the majority of laboratory-based models which has limited predictive biomarker discovery.

Overall, the findings of this thesis showed the complex nature of the response mechanisms. While it is unlikely for a single biomarker to be highly effective for detecting response and survival outcome for patients, our data demonstrated an alternative and efficient approach to predict response and survival outcome of the glioblastoma patients using a novel combination of multiple biomarkers.

Declaration relating to disposition of project thesis/dissertation

I hereby grant to the University of New South Wales or its agents the right to archive and to make available my thesis or dissertation in whole or in part in the University libraries in all forms of media, now or here after known, subject to the provisions of the Copyright Act 1968. I retain all property rights, such as patent rights. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

I also authorise University Microfilms to use the 350 word abstract of my thesis in Dissertation Abstracts International (this is applicable to doctoral theses only).



Signature

Witness

12.11.2012

Date

The University recognises that there may be exceptional circumstances requiring restrictions on copying or conditions on use. Requests for restriction for a period of up to 2 years must be made in writing. Requests for a longer period of restriction may be considered in exceptional circumstances and require the approval of the Dean of Graduate Research.

FOR OFFICE USE ONLY

Date of completion of requirements for Award:

THIS SHEET IS TO BE GLUED TO THE INSIDE FRONT COVER OF THE THESIS

COPYRIGHT STATEMENT

'I hereby grant the University of New South Wales or its agents the right to archive and to make available my thesis or dissertation in whole or part in the University libraries in all forms of media, now or here after known, subject to the provisions of the Copyright Act 1968. I retain all proprietary rights, such as patent rights. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation. I also authorise University Microfilms to use the 350 word abstract of my thesis in Dissertation Abstract International (this is applicable to doctoral theses only). I have either used no substantial portions of copyright material in my thesis or I have obtained permission to use copyright material; where permission has not been granted I have applied/will apply for a partial restriction of the digital copy of my thesis or dissertation.'



Signed

Date 12.11.2012

AUTHENTICITY STATEMENT

'I certify that the Library deposit digital copy is a direct equivalent of the final officially approved version of my thesis. No emendation of content has occurred and if there are any minor variations in formatting, they are the result of the conversion to digital format.'



Signed

Date 12.11.2012

Abstract

Glioblastomas are characterised by remarkably high resistance to chemotherapy. The majority of patients are unresponsive to standard therapy including concurrent chemo/radiotherapy and adjuvant chemotherapy with TMZ. Investigation of potential contributors of chemoresistance is needed so that patients who do not respond can avoid futile chemotherapy and be better matched to something that might work.

The aim of this thesis was to investigate factors associated with chemotherapy resistance. The expression of single biomarkers were examined, namely Topoisomerase II alpha (TopoII α), Neuro/glial2 (NG2) and MGMT and their association with response to etoposide (TopoII α -inhibitor), cilengitide ($\alpha\beta$ 3 and $\alpha\beta$ 5 integrin-inhibitor) and temozolomide, respectively. We found that the response to therapy in each case was much more complex than one drug and one biomarker. Rather, resistance is defined by the interplay of multiple proteins and genes. In response, I took a global proteomic approach to discover novel biomarkers which might be associated with response and survival outcome of the patients. Data collected using chemically-modified ProteinChip surfaces revealed significantly differentially expressed peaks between non-responders vs. responders and short-term survivors (STS) vs. long-term survivors (LTS). Biomarker panels including multiple proteins were generated. A panel of three proteins (combined predictability = 100%) overexpressed in non-responders were further identified as DEFA3, MIF and S100A8. MIF protein was up-regulated both in non-responders and STS. The expression of these biomarkers was also examined in a validation cohort (n = 66) using IHC which we are not yet able to extract entire data. All three proteins serve as components of pro-inflammatory and inflammatory tumour microenvironment and have important roles in pro-inflammatory and pro-tumorigenic

processes. These features are not taken into consideration in the majority of laboratory-based models which has limited predictive biomarker discovery.

Overall, the findings of this thesis showed the complex nature of the response mechanisms. While it is unlikely for a single biomarker to be highly effective for detecting response and survival outcome for patients, our data demonstrated an alternative and efficient approach to predict response and survival outcome of the glioblastoma patients using a novel combination of multiple biomarkers.



Publications

Sevim H., Parkinson J.F., McDonald K.L. (2011) Etoposide-mediated glioblastoma cell death: dependent or independent on the expression of its target, topoisomerase II alpha? *Journal of Cancer Research and Clinical Oncology* 137:1705-1712. DOI: DOI 10.1007/s00432-011-1046-5.

Sevim H., Chung L., Wheeler H.R., Baxter R.C., McDonald K.L. Aberrant expression of low molecular weight proteins associated with response to conventional concurrent chemoradiotherapy treatment in glioblastoma (manuscript in preparation).

Conference Abstracts

XXVIth Annual Scientific Research Meeting, November 2009 (poster presentation)

Determining Biological Factors Associated with chemotherapy response in Glioblastoma Patients

22nd Lorne Cancer Conference, February 2010 (poster presentation)

Determining Biological Factors Associated with chemotherapy response in Glioblastoma Patients

American Association for Cancer Research (AACR) 102nd Annual Meeting, April 2011 (poster presentation)

Protein Biomarker Discovery in Glioblastoma Using SELDI-TOF MS

16th Annual Scientific Meeting & Education Day of the Society for Neuro-Oncology, November 2011 (poster presentation)

Aberrant expression of low molecular weight proteins associated with response to conventional concurrent chemoradiotherapy treatment

Acknowledgments

This thesis cannot be done without support of many people. First of all, I would like to thank with greatest sincerity to my supervisor Dr Kerrie L. McDonald, for the all encouragement, advice, guidance, friendship and being a great supervisor through the entire PhD process.

I would also like to thank all members of Neuro-oncology Laboratory (Dan, Hazem, Siska, Sarita, Sylvia, Han, Hae Won, Irene, Jack and Kyoko) and Adult Cancer Program for their great support and friendship.

I greatly appreciate the level of support and help from Prof Robert C. Baxter and Dr. Liping Chung for expanding my knowledge in proteomic-based techniques.

I would like to extend a thank you to the members (Dindy, Martyn, Nicole and Sanaz) of Cancer Genetics Group in Kolling Institute of Medical Research that gave me amazing support during the first two years of my PhD process. I would like to thank Dr. Helen Wheeler. Her advice was appreciated. Thank you to Ms Sanaz Maleki and Dr Fei Shang for immunohistochemical work.

I would like to thank The Ministry of National Education, Republic of Turkey for offering me the postgraduate research scholarship.

A very special thanks to my dear friends (Selcen, Şükran, Feyza, Dilara, Eylül, Feriha and Burcu) who have all been my family away from home.

Finally, I would like to deeply thank my beloved parents, Enver and Fatma, my sister, Suadiye and my aunt, Ayşe for their love and support over the years. Their limitless love and unwavering support have been vital for me in completing this thesis.

Abbreviations

μg	Microgram
μl	Microlitre
μM	Micromolar
¹²⁵ I	Iodine-125
¹⁹² I	Iridium-192
2-DIGE	Two dimensional-difference gel electrophoresis
2-HG	2-hydroxyglutarate
5-ALA	5-aminolevulinic acid

A

ABC	ATP-binding cassette
ACN	Acetonitrile
AP	Apurinic/apyrimidinic
Apo AI	Apolipoprotein AI
Apo AII	Apolipoprotein AII
Apo CI	Apolipoprotein CI
APS	Ammonium persulfate
ATCC	American type culture collection
ATM	Ataxia Telangiectasia mutated protein
ATP	Adenosine triphosphate

B

BBB	Blood brain barrier
BCNU	1,3-bis (2-chloroethyl)-1-nitrosourea (bis-chloroethylnitrosourea)
Bcr-Abl	Break point cluster region-abelson
BER	Base excision repair
bHLH	Basic helix-loop-helix
BLR	Binary logistic regression
bp	Base pair
BSA	Bovine serum albumin

C

CA125	Cancer antigen 125
cdc	Cell division cycle
cdc42	Cell division control protein 42
CDKN2A	Cyclin dependent kinase inhibitor 2A
cdks	Cyclin-dependent kinases
cDNA	Complementary deoxyribose nucleic acid
Chk1/2	Checkpoint kinase 1 and 2
cm Hg	Centimetre of mercury
CM10	Weak cation exchange
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycan
CT	Computed tomography
CTAPIII	Connective tissue activating protein III
CV	Coefficient of variation

D

Da	Dalton
DAB	3,3-diaminobenzidine
DSB	Double strand break
DMEM	Dulbecco's modified eagles media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNA-PK	DNA dependent protein kinase
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

E

E2F	E2 promoter binding factor
ECM	Extracellular matrix
EDTA	Ethylane-diamine-tetra-acetic acid

EGF	Endothelial growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMD 121974	Cilengitide
ERBB2	Erythroblastic leukemia viral oncogene homolog 2 (HER2)
ERK1/2	Extracellular signal-regulated kinase 1 and 2

F

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
FGF-2	Fibroblast growth factor-2
FISH	Fluorescence in-situ hybridization

G

GAG	Glycosaminoglycan
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GRIP1	Glutamate receptor interacting protein
GUS β	β -Glucuronidase
Gy	Gray

H

H2AX	Histone 2AX
H50	Hydrophobic surface
HCl	Hydrochloric acid
HER2	Human epidermal receptor 2
HIFs	Hypoxia inducible factors
HPLC	High performance liquid chromatography
HREC	Human research ethics committee

I

IC ₅₀	Half maximal inhibitory concentration
ICAT	Isotope coded affinity tag
IDH	Isocytate dehydrogenase
IHC	Immunohistochemistry
IMAC	Immobilized metal affinity capture
IMS	Imaging mass spectrometry
INA	Alpha-intemexin
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4

K

kb	Kilobase
kDa	Kilodalton
KPS	Karnofsky performance status

L

LC	Liquid chromatography
LiCl	Lithium chloride
LLC	Lewis lung carcinoma
LOH	Loss of heterozygosity
LTS	Long term survived

M

m/z	Mass to charge
MADM	Mosaic analysis with double markers
MCM	Micro-chromosome maintenance
MDM2	Murine double minute 2
MDR	Multidrug resistance
MELK	Maternal embryonic leucine zipper kinase
MES	2-(N-morpholino)ethanesulfonic acid

MGMT	O ⁶ -methylguanine-DNA methyltransferase
miRNA	microRNA
MLH1	MutL homolog 1
mM	Millimolar
MMP	Metalloproteinases
MMR	Mismatch repair
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRP	Multi drug resistance associated protein
MS	Mass spectrometry
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSIA	Mass spectrometric immuno assay
MS-MLPA	Methylation specific multiplex ligation-dependent probe amplification
MSP	Methylation-specific polymerase chain reaction
MT3-MMP	Membrane-type 3 MMP
MTIC	3-methyl-(triazene-1-yl)imidazole-4-carboxamide
MTS	3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MUC-1	Mucin-1
MUPP1	Multi PDZ-binding motif

N

NABTC	North American Brain Tumor Consortium
NBORF	Human orbital frontal cortex brain total RNA
NBPCP	Human parietal cortex posterior brain total RNA
NBPCS	Human parietal cortex superior brain total RNA
NBTot	Human brain reference RNA
NF	Normalisation factor
Nf1	Neurofibromatosis type 1

ng	Nanogram
NG2	Neuroglial condroitin sulphate proteoglycan 4-neuronal/glia 2
NHEJ	Non-homologous DNA end joining
nJ	Nanojoule
NP20	Normal phase
NSCCH	Northern Sydney central coast health
NSCs	Neural stem cells

O

OCT4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte lineage transcription factor 2
OPCs	Oligodendrocyte precursor cells
OS	Overall survival

P

P14 ^{ARF}	p14 alternate reading frame
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGF-AA	Platelet derived growth factor-AA
PDGFRA	Platelet-derived growth factor receptor A
PFS6	6-month progression free survival
P-gp	P-glycoprotein
PI3K	Phosphoinositol-3-kinase
PI3KR1	Phosphoinositol-3-kinase regulatory subunit 1
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
pmol	Picomole
PMS2	Postmeiotic segregation increased 2
PSA	Prostate specific antigen
psi	Pound-force per square inch

PTEN Phosphate and tensin homolog

Q

Q10 Strong anion exchange

qRT-PCR Quantitative real-time PCR

R

rac1 Ras-related C3 botulinum toxin substrate 1

REST Relative expression software tool

RFP Red fluorescent protein

RNA Ribose nucleic acid

ROS Reactive oxygen species

RPA Replication protein A

RP-HPLC Reverse phase-high performance liquid chromatography

RP-LC Reverse phase-liquid chromatography

RT Room temperature

S

S/N Signal to noise ratio

SDS Sodium dodecyl sulfate

SELDI-TOF-MS Surface enhanced laser desorption/ionization time-of-flight mass spectrometry

SGZ Subgranular zone

SH3 Src homology 3

SHH Sonic hedgehog

siRNA Small interfering ribose nucleic acid

SPA Sinapinic acid

SQ-MSP Semiquantitative MSP

SRS Stereotactic radiosurgery

STS Short term survivors

SUMO-1 Small ubiquitin-like modifier-1

SVZ Subventricular zone

T

TBE	Tris borate/EDTA
TCGA	The cancer genome atlas
TDP1	Tyrosyl-DNA phosphodiesterase 1
TE	Tris-EDTA
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TGF- α	Transforming growth factor- α
TIC	Total ion current
TMZ	Temozolomide
TopoI	Type I topoisomerases
TopoII	Type II topoisomerases
TopoII α	Topoisomerase II alpha
Tris	Tris(hydroxymethyl) aminoethane
TT	A truncated form of transthyretin

U

UV	Ultraviolet

V

v/v	Volume (of solute) per volume (of solvent)
VEGF	Vascular endothelial growth factor

W

w/v	Weight (grams) per volume (ml)
WHO	World health organisation

X

x g	Acceleration due to gravity

Amino Acid Abbreviations

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

TABLE OF CONTENTS

ORIGINALITY STATEMENT	I
Thesis/Dissertation Sheet	II
COPYRIGHT STATEMENT.....	III
Abstract.....	IV
Publications	VI
Conference Abstracts.....	VII
Acknowledgments.....	VIII
Abbreviations.....	IX
Amino Acid Abbreviations	XVII
CHAPTER 1	1
LITERATURE REVIEW	1
1.1 BRAIN TUMOURS	1
1.1.1 Classification of Brain Tumours and Introduction to WHO grade IV Glioma	1
1.1.2 Cellular Origins of Gliomas	4
1.1.3 Genetics of Gliomas	7
1.1.4 Therapeutic Management of Glioblastomas	13
1.1.5 Prognostic Markers for Gliomas	19
1.1.6 Targeted Therapies and Novel Approaches	25
1.2 TOPOISOMERASE II ALPHA, A POTENTIAL TARGET FOR ETOPOSIDE ...	29
1.2.1 Topoisomerases	29
1.2.2 TopoII α and Anticancer Drug Interaction.....	34
1.2.3 Association between <i>p53</i> , TopoII α and response to Etoposide Treatment.....	36
1.2.4 TopoII α , Solid Cancers and Glioma	37

1.3 NG2 PROTEOGLYCAN	38
1.3.1 Neuronal/glial 2 (NG2).....	39
1.3.2 Expression of NG2 in Different Cell Types.....	41
1.3.3 Functions of NG2	42
1.3.4 NG2 Expression in Glioma	45
1.3.5 Cilengitide	46
1.4 PROTEOMICS	47
1.4.1 Definition of Proteomics	47
1.4.2 Proteomic Technologies	47
1.4.3 Applications of ProteinChip SELDI-TOF MS Technology in Pathological States	47
1.5 AIMS OF THIS THESIS.....	66
CHAPTER 2	67
MATERIALS AND METHODS	67
2.1 INTRODUCTION	67
2.2 CELL CULTURE METHODS.....	67
2.2.1. Maintenance of Cell Lines	67
2.2.2 Cryopreservation Technique	68
2.2.3 Cell Proliferation Assay.....	69
2.2.4 Cell Density Optimisation and Chemosensitivity Assays.....	70
2.2.5 Determination of IC ₅₀	71
2.2.6 Trypan Blue Cell Counting	72
2.3 DNA METHODS	73
2.4 RNA METHODS.....	73
2.4.1 RNA Extraction from Cultured Cells	73
2.4.2 Lithium Chloride (LiCl) Precipitation of RNA	74

2.4.3 cDNA Synthesis.....	75
2.4.4 Control for Genomic DNA Contamination of RNA Samples	77
2.4.5 Quantitative Real-Time PCR (qRT-PCR).....	78
2.5 PROTEIN METHODS	80
2.5.1 Protein Extraction from Glioma Cell Lines	80
2.5.2 Determining Protein Concentration	81
2.5.3 Immunoblotting	81
2.6 IMMUNOHISTOCHEMICAL STAINING METHODS	85
2.6.1 Cell Block Preparation from Cell Lines	85
2.6.2 Immunohistochemical Staining of Cell Blocks	86
2.6.3 Scoring System for Immunohistochemical Staining of Cell Blocks	87
CHAPTER 3	89
THE ROLE OF TopoIIα IN THE CELLULAR RESPONSES TO ETOPOSIDE	89
3.1 INTRODUCTION	89
3.2 METHODS	92
3.2.1 Characteristics of Glioma Cell Lines	92
3.2.2 Determining Optimal Cell Number for 96-Well Plating.....	94
3.2.3 Preparation of Etoposide	94
3.2.4 Experimental Design for Etoposide Treatment	94
3.2.5. Determining Efficiency of TopoII α Gene Expression Using RT-qPCR.....	95
3.2.6 Statistical Analysis	95
3.3 RESULTS	96
3.3.1 Determining Optimal Cell Number for 96-Well Plating.....	96
3.3.2 Establishing Endogenous Levels of TopoII α in Glioma Cell Lines	98
3.3.3 Response Characteristics of Glioma Cell Lines to Etoposide Treatment	102

3.3.4 Alterations in the Total Protein and mRNA Levels of TopoII α Post Etoposide Treatment.....	106
3.4 DISCUSSION	110
CHAPTER 4.....	116
NG2 EXPRESSION IN GLIOMA CELL LINES AND CONTRIBUTION TO DRUG RESISTANCE.....	116
4.1 INTRODUCTION	116
4.2 METHODS	119
4.2.1 Determining Efficiency of NG2 Gene Expression Using RT-qPCR.....	119
4.2.2 Immunoblotting for NG2 protein	119
4.2.3 Preparation of Cilengitide	119
4.2.4 Transfection of Cells Using Nucleofection Technique	119
4.2.5. TUNEL Assay.....	121
4.3 RESULTS	123
4.3.1 Determining Endogenous Expression Levels of NG2 in One Osteosarcoma and the Five Glioma Cell Lines and Their Responses to Cilengitide.	123
4.3.2 Knockdown of NG2 Expression in A172 Cells	127
4.3.3 Monitoring the Cilengitide-response of the Transfected and Untransfected A172 Cells	132
4.3.4 Cilengitide-induced Apoptosis in siRNA-52-transfected and -untransfected A172 Cells	136
4.3.5 Cilengitide (7, 10, 15 μ M)-induced Apoptosis in Untransfected and siRNA-52-Transfected A172 cells	142
4.4 DISCUSSION	148

CHAPTER 5	153
THE ROLE OF MGMT PROMOTER METHYLATION AND LEVEL OF PROTEIN EXPRESSION IN DETERMINING THERAPY RESPONSE AND SURVIVAL OUTCOME	153
5.1 INTRODUCTION	153
5.1.1 Temozolomide (TMZ)	153
5.1.2 MGMT	154
5.2 METHODS	157
5.2.1 Ethics.....	157
5.2.2 Clinical Data.....	157
5.2.3 Classification of Samples	157
5.2.4 Preparation of TMZ.....	159
5.2.5 DNA Protocols for Extraction and Determination of MGMT Methylation	159
5.2.6 Immunoblotting for MGMT Protein.....	171
5.2.7 Statistical Analysis	171
5.3 RESULTS	172
5.3.1. Optimisation Steps to Prepare a Soluble and the Most Effective Form of TMZ for Cytotoxicity Assays	172
5.3.2 Collection of TMZ IC ₅₀ Values for the Five Glioma Cell Lines	178
5.3.3 <i>MGMT</i> Promoter Methylation and Protein Expression in the Five Glioma Cell Lines.....	181
5.3.4 <i>MGMT</i> Promoter Methylation and Protein Expression in a cohort of 42 glioblastoma samples.....	185
5.4 DISCUSSION	192
CHAPTER 6	196
PROTEOMIC DISCOVERY OF BIOMARKERS ASSOCIATED WITH CHEMOTHERAPY RESPONSE IN GLIOMA USING SELDI-TOF MS	196
6. 1 INTRODUCTION	196

6.2 METHODS	202
6.2.1 Protein Extraction Protocols	203
6.2.2 Protein Quantification Method using <i>DC</i> TM Protein Assay	204
6.2.3 Preparation of ProteinChip Arrays for Analysis using SELDI-TOF MS	205
6.2.4 Laser Intensities and Binding Buffers	207
6.2.5 Classification of Samples	208
6.2.6 Pre-processing of Data	209
6.2.7 Statistical Analysis	210
6.2.8 Identification of Candidate Biomarkers	212
6.2.9 Validation of Identified Biomarkers by Western Blotting.....	215
6.2.10 Protocol for Immunohistochemical Staining of DEFA3, S100A8 and MIF protein expression.....	216
6.3 RESULTS	218
6.3.1 Optimisation of Lysis Buffer Used in Protein Extraction.....	218
6.3.2 Optimisation of Laser Intensities and Binding Buffers	221
6.3.3 SELDI-TOF MS Data Analysis.....	224
6.3.4 Selection of Significant Proteins and BLR Analysis	226
6.3.5 Development of Candidate Biomarker Panels by BLR Analysis in Response and Survival Cohorts.....	227
6.3.6 ROC Curve Analysis and Spearman Correlation of Three Biomarkers Up- regulated in Non-responder Patient Group	233
6.3.7 ROC Curve Analysis and Spearman Correlation of Five Biomarkers Up-regulated in Responder Patient Group	237
6.3.8 ROC Curve Analysis and Spearman Correlation of Up-regulated Biomarkers in Survival Subgroups	241

6.3.9 Protein Purification and Identification.....	245
6.3.10 Validation of Identified Biomarkers in Response and Survival Patient Cohorts by Immunoblotting	250
6.3.11 Expression of Identified Biomarkers in GNS Cultures and Glioma Cell Lines	254
6.3.12 Immunohistochemical Validation of Identified Biomarkers	256
6.4 DISCUSSION	263
CHAPTER 7.....	278
SUMMARY AND FUTURE DIRECTIONS	278
APPENDIX A.....	287
APPENDIX B.....	297
APPENDIX C	301
APPENDIX D.....	304
APPENDIX E	308
REFERENCES	312

INDEX OF TABLES

Table 2.1 MTS assay mixture for one 96 well plate.....	70
Table 2.2 SuperScript® III First-Strand Synthesis System components	76
Table 2.3 PCR conditions of cDNA synthesis	76
Table 2.4 PCR components.....	77
Table 2.5 Details of qRT-PCR assays	79
Table 2.6 Components for the qRT-PCR reaction mix.....	79
Table 2.7 Thermal cycling programs.....	79
Table 2.8 Components of protein extraction buffer.....	80
Table 2.9 Dilutions of BSA protein standard	81
Table 2.10 Components of protein mix for gel loading	82
Table 2.11 Details of electrophoresis and transfer steps for different target proteins	84
Table 2.12 Dilutions and duration of incubation for antibodies used in immunoblotting	85
Table 2.13 Allred scoring system used in assessing staining results	88
Table 3.1 Characteristics of glioma cell lines.....	93
Table 3.2 Optimum number of cells to use for cytotoxicity assays.....	96
Table 3.3 IC ₅₀ doses (µM) of glioma cell lines at 48 hr post-treatment.....	105
Table 4.1 Number of cells plated for control and transfected groups	120
Table 5.1 Clinical data of patients whose tumours were included in biomarker discovery study using SELDI-TOF MS	158
Table 5.2 MGMT PCR reagents.....	164
Table 5.3 MGMT thermal cycler conditions	164
Table 5.4 MGMT PCR reagents.....	165
Table 5.5 MGMT thermal cycling conditions.....	165
Table 5.6 MGMT PCR reagents for pyrosequencing.....	167
Table 5.7 MGMT thermal cycler conditions for pyrosequencing	167

Table 5.8 Master Mix combinations for PCR optimisation.....	169
Table 5.9 Thermal cycler conditions for GUS β DNA amplification	169
Table 5.10 Optimisation steps of TMZ used in cytotoxicity assays to determine IC ₅₀ dose of glioma cell lines	173
Table 5.11 Solubility of TMZ in different concentrations of solvents.....	173
Table 5.12 MGMT promoter methylation status analysed using pyrosequencing method in 42 glioma samples	188
Table 5.13 Summary of MGMT promoter methylation of tumour samples in response cohort	189
Table 5.14 Spearman correlation coefficients calculated to demonstrate the relationship between response characteristics of the patients and MGMT promoter methylation and MGMT protein expression detected in the patient tumour samples.....	191
Table 6.1 Summary of publications on Glioma Proteomics using SELDI-TOF MS....	199
Table 6.2 Summary of publications on glioma proteomics using SELDI-TOF MS	200
Table 6.3 Protein lysis buffer components for SELDI-TOF MS analysis.....	203
Table 6.4 Classification of samples (training cohort).....	209
Table 6.5 Buffers used for stepwise elution of crude protein samples.....	213
Table 6.6 Electrophoretic conditions used for the separation of proteins.....	215
Table 6.7 Components of protein mix for gel loading	216
Table 6.8 Conditions of staining for the antibodies used in immunohistochemistry	217
Table 6.9 Conditions optimised for laser intensities	221
Table 6.10 Optimal pH Conditions for array binding buffers	222
Table 6.11 Summary of the normalisation analysis.....	224
Table 6.12 Results for peak clustering of sample groups (Response and Survival)	226
Table 6.13 Significantly differentially expressed proteins analysed by BLR.....	227
Table 6.14 Spearman correlation for three biomarkers identified in response cohort ..	236
Table 6.15 Spearman correlation for five biomarkers identified in response cohort	240

Table 6.16 Spearman correlation for two biomarkers identified in survival cohort.....	244
Table 6.17 Summary of Mascot search results for identified proteins.....	249
Table D.1 Up-regulated proteins in non-responders.....	304
Table D.2 Up-regulated proteins in responders.....	305
Table D.3 Up-regulated proteins in STS.....	306
Table D.4 Up-regulated proteins in LTS.....	307



INDEX OF FIGURES

Figure 1.1 Molecular alterations on the progression of primary and secondary glioma.	11
Figure 1.2 Kaplan-Meier plot for overall survival comprising the two treatment groups	19
Figure 1.3 Structure of TopoII α	30
Figure 1.4 Torsional stress and formation of positive and negative DNA supercoils	33
Figure 1.5 Formation of TopoII α -DNA cleavage complex and drug induced DSBs	34
Figure 1.6 The schematic structure of NG2.....	40
Figure 1.7 SELDI technology utilise various types of modified surfaces to capture and analyse proteins with unique properties	54
Figure 1.8 Biomarker discovery in patient samples by SELDI-TOF MS using ProteinChip arrays.....	55
Figure 2.1 A sample design of cytotoxicity assays as illustrated.....	71
Figure 2.2 An example of dose-response graph demonstrating IC ₅₀ value	72
Figure 2.3 GUS β DNA amplification to control for genomic DNA contamination of the cDNA samples converted from RNAs.	78
Figure 2.4 Allred scoring system used in assessing staining results	88
Figure 3.1 Results of optimum cell number assay at 24, 48, 72 hr.....	97
Figure 3.2 Endogenous protein expression levels in a panel of five glioma cell lines ...	99
Figure 3.3 Immunohistochemical staining of glioma cell blocks for TopoII α	100
Figure 3.4 Relative mRNA expression levels measured by qRT-PCR.....	101
Figure 3.5 Response of glioma cell lines to etoposide treatment (24, 48 and 72 hr)	134
Figure 3.6 Combination of response results of cell lines treated with different dose ranges of etoposide for 48 hr	104
Figure 3.7 Levels of TopoII α pre and post etoposide treatment	139
Figure 3.8 Relative mRNA expression levels measured by qRT-PCR.....	109
Figure 3.9 Schematic illustration of the proposed mechanisms of etoposide transport in two glioma cell lines	113

Figure 4.1 A schematic model illustrating our hypothesis that NG2 plays a role in drug resistance via stimulation of downstream cell proliferation, motility and survival associated pathways	117
Figure 4.2 NG2 expression at total protein and mRNA level in the five glioma and one osteosarcoma cell line.....	125
Figure 4.3 Inhibitory effects of cilengitide on glioma cell lines and a NG2-positive control cell line (24 hr treatment).....	126
Figure 4.4 Knockdown of NG2 in the A172 cell line (24 hr)	129
Figure 4.5 Knockdown of NG2 in A172 cells at 24, 48 and 72 hr after siRNA-52 treatment	130
Figure 4.6 Parameters examined to improve endogenous expression levels of NG2 and transfection conditions.....	131
Figure 4.7 Cilengitide responses of the A172 cell groups, transfected vs untransfected and cilengitide-treated vs –untreated, detected by both trypan blue counting and MTS cell proliferation assay.....	135
Figure 4.8 TUNEL staining of NG2 knock down cells treated with cilengitide	141
Figure 4.9 Dose-dependent increase in the number of apoptotic cells in untransfected and siRNA-52 transfected A172 cells stained with TUNEL (at 24 hr after addition of cilengitide).....	147
Figure 4.10 Summary of hypothesised association between NG2, cilengitide and integrins	148
Figure 5.1 The role of MGMT in DNA repair (Hegi et al., 2008).....	155
Figure 5.2 Polyacrylamide gel electrophoresis of PCR products	170
Figure 5.3 Effect of the sterilisation method on the effectiveness of drug.....	174
Figure 5.4 Response of glioma cell lines to stock concentration of 30mM TMZ at 48 hr after addition of the drug.....	176
Figure 5.5 Response of glioma cell lines to stock concentration of 60mM TMZ at 48 hr after addition of the drug.....	177

Figure 5.6 Response of glioma cell lines to stock concentration of 90mM TMZ at 72 and 96 hr after addition of the drug	179
Figure 5.7 IC ₅₀ doses (µM) of glioma cell lines at 96 hr post TMZ treatment	180
Figure 5.8 Promoter methylation status of MGMT gene identified using MSP (A) and pyrosequencing (B) methods DNA samples of the five glioma cell lines	182
Figure 5.9 MGMT protein expression levels in the five glioma cell lines	184
Figure 5.10 MGMT promoter methylation status analysed using MSP method in 42 glioma samples	187
Figure 5.11 MGMT protein expression in 42 tumour samples	190
Figure 5.12 Kaplan Meier analysis comparing overall survival of patients with and without methylated MGMT promoter (A) and MGMT protein expression (B)	191
Figure 6.1 The workflow followed for biomarker discovery	202
Figure 6.2 Standard curve of BSA in lysis buffer without DTT	219
Figure 6.3 Standard curve of BSA in lysis buffer with DTT	219
Figure 6.4 Standard curve of BSA in lysis buffer without DTT used for the quantification of proteins from the cell lines	220
Figure 6.5 Standard curve of BSA in lysis buffer without DTT used for the quantification of proteins from the tumour tissue samples	220
Figure 6.6 Optimisation of buffers using T98G cell line protein extracts for Q10 array	222
Figure 6.7 Optimisation of buffers using T98G cell line protein extract for CM10 array	223
Figure 6.8 Candidate biomarker panels identified in patient subgroups by BLR Analysis	231
Figure 6.9 A representative overlay spectra of tumour tissue sample profiles of responders versus non-responders analysed by CM10 arrays	232

Figure 6.10 ROC curve analysis, Hanley & McNeil test including 95% CIs and representative heat map showing expression levels for the three biomarkers identified in Response cohort.....	235
Figure 6.11 ROC curve analysis, Hanley & McNeil test including 95% CIs the five biomarkers identified in Response cohort.....	239
Figure 6.12 ROC curve analysis, Hanley & McNeil test including 95% CIs the two biomarkers identified in survival cohort	243
Figure 6.13 Representative spectra for the semi-purification of proteins using Mustang S Filter Plate	245
Figure 6.14 Analysis of semi-purified fractions on NP20 arrays	247
Figure 6.15 Validation of the three identified proteins DEFA3 (A), MIF (B), and S100A8 (C) by western blotting in response cohort.....	252
Figure 6.16 Validation of MIF in survival cohort by immunoblotting	253
Figure 6.17 Expression of the three biomarkers in cell lysates of GNS and glioma cell lines	255
Figure 6.18 Expression of three biomarkers in BAH1 GNS cell lysate and conditioned medium of BAH1 GNS cells and glioma cell lines	255
Figure 6.19 Representative images of human tissues used as positive controls	257
Figure 6.20 Immunohistochemical analysis of human DEFA3 expression in tumour samples from the patients with newly diagnosed glioblastoma	258
Figure 6.21 Immunohistochemical analysis of human S100A8 expression in tumour samples from the patients with newly diagnosed glioblastoma	259
Figure 6.22 Representative immunohistochemical staining for DEFA3 and S100A8 (Higher Magnification-63X).....	260
Figure 6.23 Representative immunohistochemical staining for MIF showing distribution of MIF-positive cells.....	261
Figure 6.24 Immunohistochemical analysis of human MIF expression in tumour samples from the patients with newly diagnosed glioblastoma	262

Figure C.1 DNA ladder, pUC19/HpaII	300
Figure C.2 Precision plus protein Kaleidoscope standards.....	300
Figure C.3 HiMark* pre-stained high molecular weight protein standard	301
Figure C.4 SeeBlue [®] Plus2 pre-stained plus protein standard	301
Figure C.5 BenchMark [™] unstained plus, protein standard	302



CHAPTER 1

LITERATURE REVIEW

1.1 BRAIN TUMOURS

1.1.1 Classification of Brain Tumours and Introduction to WHO grade IV Glioma

Many systems have been suggested to classify tumours of the central nervous system (CNS). Over the decades, three criteria; cell of origin, degree of differentiation and anatomical location have been used for the classification of CNS tumours (Yates, 1992). Early in nineteenth century, suffix -oma (meaning tumour) has been added to type of cell which was believed the tumour has originated from (Yates, 1992). This system has also been employed by World Health Organisation (WHO) in grading CNS tumours. More recently, 2007 WHO classification was represented by an international group of experts including pathologists and geneticists to provide a standard global definition for tumours of CNS (Louis et al., 2007). WHO classification system mainly generates a scale of malignancy based on two principles; first, recognition of specific components of the tumour by histological examination and second, detection of similarities in protein expression using immunohistochemistry (Collins, 2004). Tumour grade

influences the treatment regime administered to patients post surgery and which can include radiotherapy and concomitant or adjuvant chemotherapy (Louis et al., 2007).

Diffuse gliomas are classified into two broad groups; astrocytomas and oligodendrogliomas which are graded from grade II to grade III and IV indicative of high grade tumours (Smith and Jenkins, 2000).

1.1.1.1 Grade II-III Gliomas

Grade II tumours are defined as diffuse, low grade gliomas. These tumours often recur after surgery because of the early infiltrative nature of tumour. They also have the potential to transform to the more malignant lesions. For example, low grade diffuse astrocytomas have a tendency toward progression of anaplastic astrocytoma (grade III) and glioblastoma (grade IV) tumours (Louis et al., 2007). Anaplastic gliomas designated grade III exhibit increased mitotic activity over type II tumours and are considered to be high grade gliomas (HGG). In most cases, radiotherapy and sometimes chemotherapy are given to the patients with grade III tumours (Louis et al., 2007). While patients with grade II tumours survive more than 5 years, grade III gliomas have a median of 2-3 years. Anaplastic gliomas can be either oligodendroglial or astrocytic and sometimes both (mixed oligoastrocytoma) (Louis et al., 2007). More than a decade ago, Cairncross and colleagues reported chemosensitivity of oligodendrogliomas and oligoastrocytomas to chemotherapy regimen including procarbazine, lomustine and vincristine (Cairncross et al., 1994). In a large cohort-based study, estimated median survival time of 11.6 years and 6.5 years were recorded for oligodendroglioma and oligoastrocytoma, respectively (Ohgaki and Kleihues, 2005).

1.1.1.2 Grade IV Glioma (Glioblastoma)

Grade IV tumours are better known as glioblastoma and are characterised by numerous malignant features which include infiltration throughout the brain parenchyma, vascular proliferation, resistance to apoptosis, uncontrolled mitotic activity. (Furnari et al., 2007; Louis et al., 2007). Presence of necrotic areas differentiates grade IV gliomas from anaplastic astrocytoma (Wen and Kesari, 2008). Glioblastoma is the most aggressive type of brain tumours and characterised by intra- and inter-tumoral histopathological, heterogeneity (Mischel and Cloughesy, 2003). Approximately, 60% of all gliomas are classified as glioblastoma (Franceschi et al., 2004). Without therapy, 95% of patients die within 3 months of diagnosis because of its infiltrative growth (Bredel et al., 2002b). In 2007, 1472 new cases of malignant brain tumours were reported in Australia (AIHW, 2010). Based on incidence/mortality data for 1998 to 2007, 1600 new cases were estimated to be diagnosed in 2010 (AIHW, 2010). Based upon the average years of life lost, brain tumours rank the highest among 17 cancer types (Burnet et al., 2005). Additionally, painful and expensive treatment procedures for glioma place a significant physical, psychological and financial burden on patients, family members and caregivers (CCA, 2011).

Glioblastoma can be diagnosed at almost any age however the median age of diagnosis is 56 years old and with modern surgical practices and chemotherapy programs, median survival of a primary glioma is 15 months (Stupp et al., 2005). The distribution between genders is dominated by men with male:female, 1:1.5 rate (Ohgaki and Kleihues, 2005; Schwartzbaum et al., 2006). The prognosis for glioblastoma varies considerably from patient to patient. In addition to WHO grading system and tumour type, there are many other parameters, such as age of the patient, neurologic performance status, location of

tumour, proliferation indices and genetic alterations that could potentially predict prognosis (Louis et al., 2007). The Karnofsky performance status (KPS) scale is used to describe the ability of patients to perform ordinary tasks and a KPS of 70 or more at diagnosis leads a favourable survival outcome (Tortosa et al., 2003).

There is not one known single cause for the occurrence of malignant gliomas. A family history of glioma has been observed approximately 4% of the patients with malignant glioma (Malmer et al., 1999). In some cases, progression of glioma has been associated with hereditary syndromes such as Li-Fraumeni (inherited p53 mutations), nevoid basal cell carcinoma, tuberous sclerosis, neurofibromatosis type 1 and 2, and adenomatous polyposis syndromes (Wrensch et al., 2002). However, a vast majority of malignant brain tumours arise in families without a known hereditary genetic predisposition (Grossman et al., 1999). Polymorphisms associated with allergies and asthma have also been shown inversely correlated with increased glioma risk (Schwartzbaum et al., 2005). In addition, exposure to therapeutic or high dose radiation has been suggested as the only well-documented external cause of glioma (Ohgaki and Kleihues, 2005).

1.1.2 Cellular Origins of Gliomas

WHO classification system suggests a cell of origin for most brain tumours, however the precise cell of origin for gliomas remain controversial. Malignant brain tumours are often formed by a combination of neuronal and glial cell types, multipotent or restricted neural progenitors and mature astrocytes (Stiles and Rowitch, 2008). The two cell of origin models; neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs) are proposed as initiators of gliomagenesis (Llaguno et al., 2009; Sugiarto et al., 2011). NSCs are primarily characterised in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus region (Li and Xie, 2005). Astrocytes and ependymal

cells that reside in SVZ and SGZ regulate self-renewal, proliferation and differentiation of NSCs. In addition, glial fibrillary acidic protein (GFAP) expressing SVZ and SGZ astrocytes function as stem cells, undergoing self-renewal and give rise to immature cells generating precursors for neuroblasts which in turn differentiate into neurons (Li and Xie, 2005). Genetic abnormalities accumulated during self-renewal of a stem cell instigate malignant stem cell transformation and generate cancer stem cells that possess diverse features such as self-renewal and resistance to chemotherapy (Reya et al., 2001). Acquired mutations cause differentiation of NCSs or glial progenitors into various neoplastic cell types exhibiting uncontrolled proliferation independent of growth signals or resistance to antigrowth signals (Li and Neaves, 2006). Expression of NSC surface marker CD133 in isolated tumour stem cells has been linked with differentiation ability of stem cells into the phenotypically diverse types of cells that generate tumour initiating cell populations similar to those found in the initial glioblastoma (Yuan et al., 2004). Due to potential contribution of CD133 in tumour initiation and recurrence, expression of CD133 has been suggested to be a prognostic predictor for poor clinical outcome (Cheng et al., 2009). Bao and co-workers reported that CD133⁺ cells isolated from glioblastoma specimens express high levels of vascular endothelial growth factor (VEGF), which further contribute to tumour initiating capacity of CD133⁺ cells and indicate possible interaction between NSCs and microvascular proliferation (Bao et al., 2006).

Numerous other proteins and signalling pathways such as octamer-binding transcription factor 4 (OCT4), oligodendrocyte lineage transcription factor 2 (OLIG2), hypoxia inducible factors (HIFs), sonic hedgehog (SHH) and Notch pathways play important roles in tumour proliferation, as well as maintenance of non-neoplastic stem cells (Li et

al., 2009). OCT4 has been reported to be a specific target of HIF2 α induced in hypoxic conditions (Covello et al., 2006). Knockdown of HIF2 α expression leads to a decrease in expression and transcriptional activity of OCT4 which further result in reduced number of embryonic primordial germ cells due to enhanced cellular differentiation (Covello et al., 2006). The Notch signalling pathway has also been investigated intensively for its potential roles in differentiation and de-differentiation of cells functioning in tumorigenesis process of glioma (Stockhausen et al., 2010). Notch signalling promotes transformation of nestin-positive glioma cells into three neural cell lineages; neuron, astrocyte or oligodendrocyte with stable nestin-expressing NSC phenotype. Moreover, formation of stem-like cells suggests that Notch signalling might promote tumorigenicity via direct modulation of tumour initiating stem cells (Zhang et al., 2008).

OLIG1 and *OLIG2* genes, two members of basic helix-loop-helix (bHLH) family have been identified in OPCs and also in zone of neuroepithelium of the spinal cord from which oligodendrocytes emerge (Zhou et al., 2000). In addition to this, OPCs can be characterised with coexpression of platelet-derived growth factor receptor A (PDGFRA) and neuroglial condroitin sulphate proteoglycan 4 (NG2) (Chang et al., 2000). NG2 expressing cells from murine OPCs and OPC-like tumour cells from transgenic mice and human oligodendrogliomas have recently been indicated as the cellular origin of oligodendrogliomas (Persson et al., 2010). This study also demonstrated that OPCs and OPC-like tumour cells were highly sensitive to temozolomide (TMZ) when compared to murine NSC and human astrocytoma cultures (Persson et al., 2010). Hence, chemosensitivity of OPCs has been associated with therapy-responsive nature of oligodendrogliomas that is thought to arise from these progenitor cells (Persson et al.,

2010). Liu and colleagues investigated the cell of origin of glioma caused by mutations inactivating neurofibromatosis type 1 (*Nf1*) and *Trp53*, mouse p53 gene, using mosaic analysis with double markers (MADM) in mouse models (Liu et al., 2011). The researchers then generated MADM-labelled cells in NSC-derived cell lineages (neurons, astrocytes, oligodendrocytes and OPCs) and created green fluorescent protein (GFP) labelled cells in a specific cell type with homozygous mutations in *Nf1* and *Trp53* and red fluorescent protein (RFP) labelled wild-type daughter cells. They observed minor growth of GFP+ NSCs, astrocytes, neurons and oligodendrocytes, while GFP+ OPCs increased by more than 130-fold when compared with RFP+ OPCs (Liu et al., 2011). Further research of this group indicated that OPCs represented the major proliferative cell type harbouring mutant *Nf1* and *Trp53* genes with pretumorigenic features and can be suggested as the cell of origin for glioma tumorigenesis (Liu et al., 2011). These data argue that gliomas involve a complex molecular and cellular diversity, therefore, identification of precise cell of origin remains to be a major obstacle to develop effective therapeutic modalities (Huse and Holland, 2010).

1.1.3 Genetics of Gliomas

Two subtypes of glioma; primary and secondary gliomas are classified depending on their different genetic progression pathways and age distribution. Primary gliomas develop *de novo* with no clinical or histological evidence of a lower grade lesion. Primary glioma cases are mostly observed with some molecular alterations, such as loss of heterozygosity (LOH) of chromosome 10 (10q), phosphatase and tensin homolog (*PTEN*) mutations, epidermal growth factor receptor (*EGFR*) and murine double minute 2 (*MDM2*) amplification (see Figure 1.1) (Furnari et al., 2007). Secondary gliomas arise from lower grade astrocytomas. For example, within 5-10 years of

diagnosis, ~70% of grade II gliomas transform into grade III/IV lesions. Inactivation of *p53* is the most frequent and earliest detectable mutation for secondary gliomas (Furnari et al., 2007). *p53* as a tumour suppressor gene negatively regulates proliferation of cells and plays an important role in transcriptional activation or repression of several genes, in particular *p21* which is an inhibitor of cyclin-dependent kinases (CDKs). Through this pathway, *p53* mediates cell cycle control at G1/S and G2/M checkpoints (Agarwal et al., 1995). Mutations in the *p53* gene have been shown in approximately 50% percent of human tumours including brain, breast, colon, lung, oesophagus and liver (Hollstein et al., 1991). However, these mutations do not necessarily reflect the inactivated transcriptional status of *p53*. Kato and co-workers reported that only 15.6% of mutant *p53* genes could fully inactivate proteins. Variable levels of proteins were expressed by 27.5% of the *p53* mutants whilst no alteration in *p53* expression was reported for the remaining 56.9% of the mutants (Kato et al., 2003). It has also been demonstrated that mutant *p53* gene lose its ability to control Topoisomerase II alpha gene promoter which results in unstrained cell division (Sandri et al., 1996). Mutations in *p53* gene have been associated with chemoresistance in many studies, such as osteosarcoma (Asada et al., 1999), breast (Berns et al., 2000), ovarian (Righetti et al., 1999) cancers. Coimmunoprecipitation of Murine Double Minute-2 (MDM-2) protein with *p53* has been reported to show MDM-2 mediated inactivation of *p53* protein which leads to ubiquitin-dependent proteasomal degradation (Momand et al., 1992; Yang et al., 2004b). MDM-4 is another key negative regulator of *p53*. *MDM-2* and *MDM-4* has been evidenced to be amplified in 10-20% of human cancers, specifically with 10.4 and 4.6-11.5% amplification rate for *MDM-2* and *MDM-4* in brain tumours, respectively (Toledo and Wahl, 2006). Isocitrate dehydrogenase 1 (*IDH1*) and *IDH2*

mutations are recently characterised as a feature of Grade II; astrocytic, oligodendroglial and oligoastrocytic gliomas. Anaplastic astrocytomas (grade III) carry additional genetic alterations such as deletions on chromosomes 6p, 9p, 11p and 22q. Mutations on 9p result in loss of function in relevant target tumour suppressor genes cyclin dependent kinase inhibitor 2A (*CDKN2A*), *CDKN2B* and p14 alternate reading frame (*p14^{ARF}*) (see Figure 1.1) (Riemenschneider et al., 2010). Additional mutations involving loss of heterozygosity (LOH) on 10q25-qter (~70%) cumulate in the progression process to grade IV glioma (Furnari et al., 2007; Kleihues and Ohgaki, 1999; Ohgaki et al., 2007; Ohgaki et al., 2004). Alterations of cell cycle regulatory genes cause the loss of control at checkpoints of cell division that lead to inappropriate cell division (Furnari et al., 2007).

In 2005, The Cancer Genome Atlas (TCGA) project was initiated to discover genetic alterations in large cohorts of grade IV glioma specimens. Analysis of genomic copy number alterations, CpG methylation gene expression, messenger RNA (mRNA) expression and microRNA (miRNA) expression profiles using multiple array platforms was performed in more than 200 glioma samples (Chin et al., 2008). In addition to this, 601 genes were sequenced in 91 glioma samples to catalogue mutations of glioblastomas. In the first publication of TCGA in 2008, researchers reported frequently mutated genes; epidermal growth factor receptor (*EGFR*), neurofibromin 1 (*NF1*), erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) and phosphoinositide-3-kinase regulatory subunit 1 (*PI3KR1*) and confirmed mutations on *TP53* and *RB1* genes (Chin et al., 2008). One of the most prominent findings of TCGA study is identification of mutations in at least one of the mismatch repair (MMR) genes; mutL homolog 1 (*MLH1*), mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*) or postmeiotic segregation

increased 2 (*PMS2*) along with methylated O⁶-methylguanine-DNA-methyltransferase (*MGMT*) promoter in seven recurrent tumour samples from treated (four patients treated with TMZ; three patients treated with lomustine) glioblastoma patients (Chin et al., 2008). Thus, the TCGA study revealed a hypermutator phenotype which defines patients displaying methylated *MGMT* promoter together with defects in MMR genes and suggested a potential link between patients with hypermutator phenotype and their response characteristics to therapy (Chin et al., 2008). More recently, Verhaak et al. reported four distinct subtypes of glioblastoma; proneural, neural, classical and mesenchymal, using gene expression signatures of samples in TCGA data sets (Verhaak et al., 2010). A similar classification of tumour samples as proneural, proliferative and mesenchymal molecular subtypes has been described by Phillips and co-workers in 2006. (Phillips et al., 2006). No association was reported when therapy response of glioblastoma patients were correlated with *MGMT* methylation status of samples in four molecular subtypes (Verhaak et al., 2010). While there was not any significant survival difference reported between subtypes, it has been indicated that patients with proneural GBM tended to survive slightly longer than patients classified in other subtypes (Verhaak et al., 2010). Studies integrating large tumour cohorts with novel analysing technologies might help elucidate potential contributors in molecular pathways associated with therapy resistance mechanisms (Chin et al., 2008; Phillips et al., 2006).

Figure 1.1 Molecular alterations on the progression of primary and secondary glioma

WHO grade IV gliomas either arise *de novo* (primary glioma) or progress from the lower grade astrocytic, oligodendroglial and oligoastrocytic gliomas (secondary glioma). Progression pathways involve numerous chromosomal and genetic alterations, specifically receptor tyrosine kinase / mitogen-activated protein kinase/phosphatidylinositol 3 kinase pathway RTK/MAPK/PI3K (eg *EGFR*, *MET*, *PDGFRA*, *ERBB2*, *NF1*, *PTEN*, *PIK3R1*, *PI3KCA*, *CTMP*), the p53 pathway (eg *TP53*, *p14^{ARF}*, *MDM2*, *MDM4*) and the pRb1 pathway (eg *CDKN2A*, *CDKN2B*, *CDK4*, *CDK6*, *RB1*). Primary gliomas are characterised by monosomy 10, trisomy 7 and gains of 19q and 20q. In addition, astrocytic, oligodendroglial, oligoastrocytic gliomas and secondary gliomas often harbour *IDH1* or *IDH2* mutations. WHO grade II diffuse astrocytomas carry mutations of *TP53*, whereas loss of 1p/19q is associated with oligodendroglial tumours. Molecular alterations on 9p21 resulting in inactivation of tumour suppressor genes *CDKN2A*, *p14ARF* and *CDKN2B* occur in the malignant progression pathway to anaplastic glioma. Point mutation of the *BRAF* gene on 7q34 characterises pilocytic astrocytomas.

This figure was redrawn from 'Molecular diagnostics of gliomas: state of the art' (Riemenschneider et al., 2010)

Abbreviations: BRAF: v-raf murine sarcoma viral oncogene homolog B1, CDK4: cyclin-dependent kinase 4, CDKN2A/2B: cyclin dependent kinase inhibitor 2A/2B, CTMP: *Carboxyl-terminal modulator protein*, DCC: deleted in colorectal carcinoma, EGFR: epidermal growth factor receptor, ERBB2: Erythroblastic Leukemia Viral Oncogene Homolog 2, HDM2: human double-minute 2, IDH1: isocitrate dehydrogenase 1, p14^{ARF}: p14 alternate reading frame, PDGFR: platelet-derived growth factor receptor, PI3KCA: *Phosphatidylinositol 3-kinase catalytic alpha polypeptide*, PI3KR1: *Phosphatidylinositol 3-kinase regulatory alpha subunit*, PTEN: phosphatase and tensin homologue deleted on chromosome ten, RB1: retinoblastoma susceptibility protein, WHO: World Health Organization

1.1.4 Therapeutic Management of Glioblastomas

A multidisciplinary approach including neuroradiology, neurosurgery, neuropathology, neurology, radiation and medical oncology is required for the current management of malignant gliomas (Preusser et al., 2011). The standard treatment of glioblastoma involves maximal safe surgical resection and concurrent radiotherapy with TMZ, followed by adjuvant TMZ (Clarke et al., 2010). However, glioblastoma recurs eventually in all patients despite these aggressive, multimodal treatments (Kostron and Bauer, 2011). It is also problematic in patients with gliomas that repeat surgery may not be achievable because of the infiltrative feature of the tumour. Furthermore, an additional radiation therapy would cause increased risk of the neurologic toxicity. Because of these reasons, observed progress in surgery and adjuvant therapies is still not the remedy for patients with high-grade gliomas (Franceschi et al., 2004).

1.1.4.1 Surgery

The goal of surgery in glioblastoma is to remove as much of the tumour as possible without causing damage on eloquent regions of the brain; sensorimotor, language and visual cortex, the hypothalamus, thalamus, internal capsule, brainstem, deep cerebellar nuclei and cerebellar peduncles (Nakaji et al., 2005; Weller, 2011). Tumours that reside within non-eloquent areas are resectable, whereas resection of tumours in eloquent areas remains challenging due to infiltrative growth of the tumour (Werndle et al., 2011). Although there is some evidence that no detectable improvement on KPS scores has been observed in 58 to 78 % of patients after surgery, tumour resection usually reduces effects of tumour mass and relieves symptoms (Sawaya et al., 1998). In addition, the greater extent of tumour resection has been associated with longer survival time as an independent prognostic factor (Lacroix et al., 2001; Nomiya et al., 2007). Similarly, efficacy of post-operative adjuvant therapies has been shown to be affected by the

amount of tumour remaining after surgery (Keles et al., 2004). In recent years, intra-operative use of fluorescent dye, 5-aminolevulinic acid (5-ALA) which serves as a natural biochemical precursor of haemoglobin leads to accumulation of fluorescent porphyrines in malignant glioma cells, therefore, enables detection of tumour boundary fluorescence using modified surgical microscopes and improve efficiency of surgical therapy (Stummer et al., 1998). Fluorescence-guided technology achieved a complete tumour resection in 65% of patients compared to conventional surgical operation resulted in gross total resection of only 35% of patients (Stummer et al., 2006). Whilst the use of 5-ALA indicated no alteration in overall survival time, 20% difference in 6-month progression free survival (PFS6) has been reported between fluorescent-guided and conventional surgery groups (Stummer et al., 2006).

The diagnosis of glioma is facilitated by computed tomography (CT) and magnetic resonance imaging (MRI) (Harbaugh and Black, 1998). However, the presence or absence of contrast enhancement of the tumour on CT or MRI alone is not sufficient to confirm the diagnosis. 14 to 45% of nonenhancing supratentorial gliomas were found to be malignant while some of the benign gliomas such as pilocytic astrocytoma enhance following an injection of MRI contrast (Scott et al., 2002). Surgery is therefore necessary to obtain tissue for histopathological examination and correct diagnosis of tumour type (Carapella et al., 2011). In some instances where surgery is not applicable due to tumour location, advanced age, severe comorbidities, a stereotactic biopsy might be the only option to determine the histology of tumour for developing accurate treatment approaches (Nieder et al., 2005). Despite significant advances in neurosurgery techniques such as intra-operative imaging, and fluorescence-guided surgery, a true complete surgical removal of high grade gliomas is not feasible (Carapella et al., 2011; Tonn and Stummer, 2008; Willems et al., 2006)

1.1.4.2 Radiotherapy

Following surgery, radiotherapy plays a critical and essential role in glioma treatment in particular to treat inoperable tumours (Beauchesne et al., 2010). Conventional fractionated radiotherapy delivers a 60 Gy complete dose of partial-field external beam irradiation over multiple sessions (Ferguson, 2011; Laperriere et al., 2002). Post-operative radiotherapy has been shown to be associated with prolonged median survival independent of age or KPS status in glioma patients (Filippini et al., 2008). Over the last two decades, hyperfractionated and hypofractionated therapy regimens were studied in order to assess efficacy of reduced radiation dose or dose intensification per fraction and improve outcomes of patients (Curran et al., 1993; Gupta and Dinshaw, 2005; Slotman et al., 1996). Several techniques are being used in glioma treatment. Stereotactic radiosurgery (SRS) differs from conventional external beam radiotherapy by utilising multiple convergent beams to deliver high doses of radiation to a small tumour volume while limiting the dose of radiation to the surrounding sensitive normal tissues (Cloughesy, 2011). Souhami et al. demonstrated no improvement of survival in glioma patients treated with SRS followed by conventional radiotherapy and chemotherapy with carmustine compared to patients received only conventional radiotherapy with carmustine (Souhami et al., 2004). Intestinal brachytherapy is a stereotactic radiotherapy that involves implantation of low dose radioactive pellets of iodine-125 (^{125}I) and iridium-192 (^{192}I) directly into tumour site (Vitaz et al., 2005). In a study treated 40 recurrent glioblastoma patients with maximum safe surgical resection followed by permanent ^{125}I implantation (a total dose of 120 and 160 Gy) into tumour bed, researchers reported no radiation necrosis or injury observed in patients and a median survival of over 11 months (Patel et al., 2000). Boron neutron capture therapy is a tumour selective radiotherapy based on the uptake of boron by tumour cells and a

consequent irradiation of the tumour site with neutron beam that interacts with boron (Yamamoto et al., 2008). More research is required to establish superiority of these experimental techniques to conventional therapies.

1.1.4.3 Chemotherapy

Chemotherapy is given to the glioblastoma patients either as concurrent with or/and as an adjuvant to conventional radiotherapy or as a salvage therapy for patients with recurrent glioblastoma (Chamberlain, 2006). Before 2005, the addition of cytotoxic chemotherapy regimens to the standard treatment remained controversial due to its significance on clear survival benefit (Fine et al., 1993; Stewart, 2002). There are a number of difficulties encountered in chemotherapy of glioblastoma.

First problem is the presence of intact brain endothelial capillaries often referred to as the blood brain barrier (BBB) which limits the penetration and effective delivery of the therapeutics to the brain tumour or tumour associated cells (de Vries et al., 2006; Wolburg and Lippoldt, 2002). In most cases, BBB allows transport of small molecules with molecular mass less than 400-500 Da and the drugs generating less than 8-10 hydrogen bonds with solvent water (Pardridge, 2005). In order to overcome this obstacle, administration of high-dose intravenous and intraarterial chemotherapy has been examined in glioma patients, but these trials resulted in enhanced systemic toxicities (Ashby and Shapiro, 2001; Blakeley, 2008). Nevertheless, Fortin *et al.* demonstrated that application of intraarterial infusion with and without osmotic BBB disruption has produced promising results for patients with multiple cerebral metastasis of ovarian carcinoma, lung adenocarcinomas and systemic lymphoma (Fortin et al., 2007).

Multidrug resistance (MDR) mechanisms are the second major obstacle that confers resistance to chemotherapeutic reagents by lowering their intracellular concentrations and reversing the cytotoxic effect of chemotherapeutic drugs (Fojo and Bates, 2003). There are two categories of drug resistance known as intrinsic and acquired resistance. Intrinsic resistance are commonly mediated by overexpression of ATP-binding cassette (ABC) family transporters, P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) which originally exists without previous exposure to drugs (Agarwal et al., 2011; Gottesman and Pastan, 1993). However, in acquired resistance, cells initially represent a responsive phenotype which subsequently becomes resistant during repetitive chemotherapy exposure (Gottesman and Pastan, 1993). P-gp functions as an ATP-dependent transmembrane drug efflux transporter which decreases intracellular accumulation of drug (Endicott and Ling, 1989). MDR-associated protein 1 (MRP1) is another major component of MDR mechanisms that also acts as a drug efflux pump (Agarwal et al., 2011). Both P-gp and MRP1 overexpression have been suggested to be involved in intrinsic or acquired drug resistance in gliomas (Abe et al., 1998; Abe et al., 1994; Decleves et al., 2002).

In addition to drug resistance caused by BBB and overexpression of membrane transporters, additional complexity is added to these mechanisms with the contribution of DNA repair systems such as MGMT which removes alkyl adducts (O⁶-methylguanine) generated by alkylating chemotherapy drugs (Gillet and Gottesman, 2010; Kaina et al., 2007). Temozolomide (TMZ) is an alkylating agent which has an tremendous bioavailability following oral administration and capability of being transported across the BBB (Stupp et al., 2001). In a study involving two glioma cell lines with TMZ-acquired resistance, MGMT expression was associated with resistance to TMZ (Zhang et al., 2010). However, inconsistent relationship between

heterogeneous MGMT expression in glioblastoma specimens and glioma cell lines and TMZ resistance has also been reported and suggested that MGMT might not be the only intrinsic factor in predicting response to TMZ (Juillerat-Jeanneret et al., 2008). Hegi and colleagues showed that epigenetic silencing of the *MGMT* gene by methylation of gene promoter is an independent prognostic factor of favourable outcome (Hegi et al., 2005). In 2005, Stupp *et al.* published results of a large phase III study recruited 573 newly diagnosed glioblastoma patients who were randomised to either radiotherapy alone or radiotherapy plus TMZ (Stupp et al., 2005). In this study, it has been demonstrated that concurrent TMZ and radiotherapy compared with radiotherapy alone prolonged median survival time from 12.1 months to 14.6 months. While median survival time was improved 2.5 months, two year survival was increased by 16.1% in patients receiving combination of radiotherapy and TMZ. Treatment regimen used in this study included daily TMZ dose 75 mg/m^2 administered orally, concomitant with radiotherapy. Four weeks following radiotherapy patients were treated with TMZ dose $150\text{-}200 \text{ mg/m}^2$ for five days every 28 days up to six months. This protocol is now considered as standard treatment of glioblastoma (see Figure 1.2) (Stupp et al., 2005).

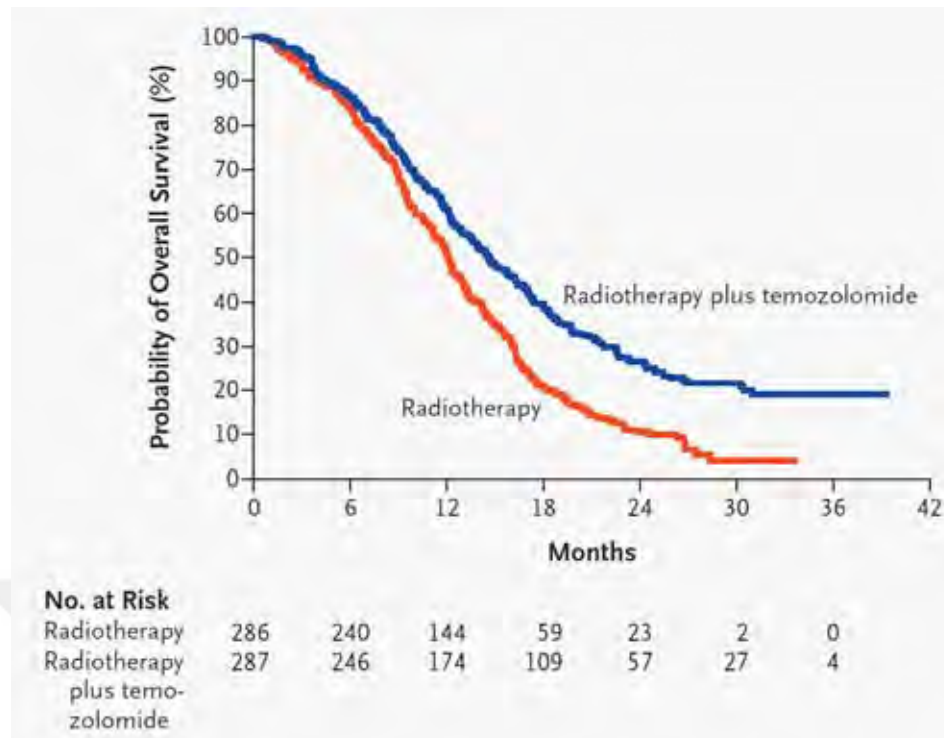


Figure 1.2 Kaplan-Meier plot for overall survival comprising the two treatment groups

Survival of glioblastoma patients randomised to radiotherapy alone or radiotherapy plus TMZ treatment groups. An established median survival benefit of 2.5 months in favour of the patients treated with TMZ has been shown by Stupp *et al.* (Stupp *et al.*, 2005).

1.1.5 Prognostic Markers for Gliomas

A prognostic biomarker provides valuable information about the overall outcome of the patients, whereas, a predictive biomarker utilises prediction of outcome after a specific treatment (Oldenhuis *et al.*, 2008). The three most significant prognostic biomarkers; codeletion of chromosome arms 1p and 19q, *MGMT* promoter methylation status and mutations in isocitrate dehydrogenase genes (*IDH1/IDH2*) have been recognised as the predictors of overall survival in gliomas.

1.1.5.1 Codeletion of chromosome arms 1p and 19q

Codeletion of chromosome arms 1p and 19q (1p/19q) is an anomaly observed in a large number of oligodendrogliomas, anaplastic oligodendrogliomas and also in oligoastrocytomas and anaplastic oligoastrocytomas (Bromberg and van den Bent, 2009). Cairncross and colleagues reported that the combined 1p/19q loss was strongly associated with a favourable response to procarbazine, lomustine and vincristine as well as radiotherapy (Cairncross et al., 1998). There is some contradiction in confirming prognostic potential of 1p/19q status. While 1p/19q loss was suggested not to be an effective prognostic factor for PFS in the grade II and III oligodendroglial tumours who received no chemotherapy and radiotherapy (Weller et al., 2007), another study demonstrated that the grade II gliomas with 1p/19q codeletion grow considerably slower than the tumour with non-codeleted 1p/19q (growth rate of 3.4mm versus 5.9mm/year); suggesting that 1p/19q presents a survival advantage to the patients by slow progression the tumour (Ricard et al., 2007). In contrast to the complete loss of 1p/19q chromosomal arms known as a common feature of oligodendroglial tumours associated with favourable outcome, partial loss of chromosome 1p occur mainly in astrocytic tumours is linked to poor prognosis (Ichimura et al., 2008; Idbaih et al., 2005). Fluorescence in-situ hybridization (FISH) is a widely used method to detect 1p/19 codeletion in addition to a few more potent techniques such as genomic arrays-comparative genomic hybridization array- and arm specific chromosome painting which both can analyse partial 1p or whole 1p/19q deletions more specifically (Griffin et al., 2006; Jenkins et al., 2006). The majority of oligodendrogliomas harbouring 1p/19q codeletions were also found to be methylated at the MGMT promoter frequently and harbour mutations in *IDH1/IDH2* and *p53* (Bourne and Schiff, 2010; Idbaih et al., 2008). In 1p/19q codeleted tumours, hypermethylated MGMT promoter and higher

percentage of methylated CpG sites were observed more frequently than 1p/19q intact tumours (Brandes et al., 2006; Mollemann et al., 2005). In a microarray gene expression based study, 1p/19q codeleted gliomas were part of a proneural gene expression profile which was linked to the relatively chemosensitive nature of proneural subtype of glioblastomas (Ducray et al., 2008; Ferrer-Luna et al., 2009). Ducray *et al.* investigated the association of 1p/19q codeletion with protein expression levels of a neuronal intermediate filament protein, Alpha-intemexin (INA) which is known as the most differentially expressed gene among proneuronal genes in gliomas. Results of this study indicated that positive expression of INA was directly correlated with the presence of 1p/19q codeletion and associated with improved PFS of 52.6 vs. 8.7 months and OS of 121.1 vs. 31.4 months for INA positive tumours versus negative tumours, respectively (Ducray et al., 2009). In a recent EORTC trial including 92 patients with oligoastrocytoma and anaplastic oligoastrocytoma, a strong correlation between INA expression and 1p19q codeletion, mutated *IDH1* and *MGMT* promoter methylation was observed suggesting INA to be a significant prognostic factor as well as 1p/19q in low grade gliomas (van den Bent et al., 2011).

1.1.5.2 IDH1 and IDH2 mutations

IDH (isocitrate dehydrogenase) is a Krebs cycle enzyme that catalyses oxidative decarboxylation of isocitrate to α -ketoglutarate and reduces NADP⁺ to NADPH (Lee et al., 1995; Xu et al., 2004). NADPH, a cofactor of glutathione reductase, is required for the reduction reaction of oxidised glutathione to glutathione. Glutathione binds reactive oxygen species (ROS) and plays an antioxidant role. Production of NADPH and therefore IDH activity is crucial for protecting cells against oxidative damage (Meister, 1988; Scrutton et al., 1990). Point mutations at the arginine 132 (IDH1) and at arginine 140 or arginine 172 (IDH2) in the active site of IDH enzyme alter catalytic activity of

the enzyme and results in increased production of 2-hydroxyglutarate (2-HG) which is potentially associated with increased cancer risk and glioma progression (Dang et al., 2009). A recent study showed that 2-HG also had a potential to competitively inhibit α -ketoglutarate-dependent enzymes including histone demethylases and 5-methylcytosine hydroxylases which in turn give rise to genome-wide histone modifications and DNA methylation changes (Xu et al., 2011). Using high-throughput next-generation sequencing, recurrent mutations in the active site of IDH1 have been recently identified in approximately 12% of glioblastomas and had a higher incidence (83%) in secondary glioblastomas compared to primary glioblastomas (7%) (Parsons et al., 2008). Mutations of *IDH1* and *IDH2* genes have been observed in a large number of patients (>70%) with WHO grade II and III astrocytomas, oligodendrogliomas and secondary glioblastomas. In this study it has also been reported that *IDH1* and *IDH2* are the only mutations present in the majority of astrocytomas and oligodendrogliomas, suggesting that these mutations can be considered as early-stage changes occurring in development of gliomas (Yan et al., 2009). Occurrence of *IDH1* mutation has been reported to be inversely associated with increasing tumour grade; *IDH1* mutation has been detected in 77% of grade II, 55% of grade III and 6% of grade IV gliomas (Sanson et al., 2009). Interestingly, pilocytic astrocytomas, ependymomas and medulloblastomas have been found to lack *IDH1* and *IDH2* mutations (Balss et al., 2008; Yan et al., 2009). Lebusiere and colleagues evaluated co-presence of *IDH* and 1p/19q mutations in glioma samples and identified *IDH* mutations in 92% of 1p/19q codeleted glioma samples (Labussiere et al., 2010). Similar to these findings, a significant ($p < 0.001$) correlation has been shown between *IDH* mutated phenotype and methylated *MGMT* promoter (Sanson et al., 2009). The clinical value of *IDH* mutations as a prognostic factor was assessed in the NOA-04 phase III clinical trial in newly diagnosed anaplastic

gliomas. The results of this study demonstrated that *IDH* mutations alone possessed more effective prognostic potential than *MGMT* promoter methylation and 1p/19q codeletion suggesting that *IDH* mutations can be utilised as a prognostic marker for anaplastic astrocytoma (Wick et al., 2009).

1.1.5.3 MGMT

DNA alkylating agents such as TMZ and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) generate an alkylated form of guanine which is referred to as O6-methylguanine (Drablos et al., 2004). MGMT removes the methyl group from O6-methylguanine and restores integrity of DNA (Pegg, 2000). Methylation of the CpG island in the MGMT promoter is suggested to be associated with a significant loss of MGMT protein expression in several tumour tissues including gliomas, colon carcinomas and lymphomas (Esteller et al., 1999). Low levels of MGMT protein expression in tumour specimens from patients with low-grade astrocytoma, malignant astrocytoma and glioblastoma have been shown to be an independent prognostic factor of better OS (Friedman et al., 1998; Jaeckle et al., 1998). A number of other studies have also shown significant correlations between *MGMT* methylation and responsiveness to chemotherapy and better prognosis (Esteller et al., 2000; Hegi et al., 2005; Paz et al., 2004).

Hegi and colleagues investigated the predictive and prognostic potential of *MGMT* promoter methylation in two treatment groups of glioma patients treated with concomitant and adjuvant TMZ versus patients receiving radiotherapy alone (Hegi et al., 2005). Patients with methylated *MGMT* promoter and had undergone TMZ treatment showed a significant survival advantage compared to the patients treated with radiotherapy alone. No clear survival benefit was reported in patients with unmethylated

MGMT promoter (Hegi et al., 2005). In 2009, Stupp *et al.* reported the 5 year median follow-up results of 573 patients with newly diagnosed glioblastoma recruited in EORTC-NCIC trial which was designed to assess the impact of *MGMT* promoter methylation status on overall survival of patients receiving radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone (Stupp et al., 2009). In this study, combined treatment with TMZ provided a significant survival advantage to the patients with methylated *MGMT* promoter (48.9% survived 2 year) compared to the patients with unmethylated *MGMT* promoter (14.8% survived 2 year) (Stupp et al., 2009). Therefore the methylation status of *MGMT* promoter was suggested to be an encouraging predictor of response to alkylating chemotherapy. However, NOA-04 phase III trial conducted in anaplastic glioma patients demonstrated that patient response to alkylating agents was not simply predicted by the methylation status of *MGMT* promoter, although it was found to be potent prognostic factor (Wick et al., 2009). van den Bent and colleagues have also reported similar findings suggesting that *MGMT* promoter methylation was not predictive for outcome in patients with oligodendroglial tumours treated with procarbazine, lomustine and vincristine chemotherapy (van den Bent et al., 2009a). While these studies exhibit a robust prognostic role of *MGMT* promoter methylation in treatment strategy of gliomas, its predictive potential needs further investigation due to some controversial findings reported by multiple glioma studies.

MGMT promoter methylation status is currently determined by several available methods including methylation specific PCR (MSP), semiquantitative MSP (SQ-MSP) and pyrosequencing (Everhard et al., 2009; Jeuken et al., 2007). In a more recent study, Karayan-Tapon et al. examined *MGMT* promoter methylation in glioblastoma patients using MSP, SQ-MSP and pyrosequencing. These researchers suggested that the most

effective technique to predict overall survival was pyrosequencing which allows identification of partial methylation and produces quantitative results by generating percent methylation for each CpG site in the sequence analysed (Karayan-Tapon et al., 2010; Mikeska et al., 2007). Therefore, the use of a standardised *MGMT* promoter methylation test in particular pyrosequencing could facilitate determining prognosis of glioma patients with glioma. It is noteworthy that numerous studies have failed to show a correlation between *MGMT* methylation and protein expression (Brell et al., 2011; Cao et al., 2009; Preusser et al., 2008; Tang et al., 2011).

1.1.6 Targeted Therapies and Novel Approaches

The main goal of targeted therapies is to render inhibition of tumour growth by selectively targeting multiple signalling pathways including Epidermal growth factor receptor (EGFR), Platelet-derived growth factor receptor (PDGFR), Vascular endothelial growth factor (VEGF) and integrins and their downstream effectors such as PI3K/Akt and Ras/Raf/MEK/MAPK pathways which altogether play important roles in tumour progression (Mischel and Cloughesy, 2003). The role of these potential therapeutic targets in key signalling pathways is briefly discussed below. EGFR is a transmembrane tyrosine kinase which belongs to the Erythroblastic leukemia viral oncogene homolog (ERBB) growth factor receptor family. A number of different ligands such as endothelial growth factor (EGF), transforming growth factor- α (TGF- α), heparin-binding EGF, epiregulin and neuregulin G2b exert their functions via binding to the EGFR. Activation of the EGFR pathway plays an important role in mitogenesis, apoptosis, cellular motility and angiogenesis (Bianco et al., 2006). *EGFR* gene amplification has been observed in approximately 40-50% of glioblastomas (Dunn et al., 2000). Platelet derived growth factor ligands (PDGFA, B, C and D) bind to receptor tyrosine kinases, PDGFR- α and - β and phosphorylation of PDGFRs activates

downstream pathways which prompts proliferation, cellular migration and angiogenesis (Board and Jayson, 2005; Heldin, 1992). VEGFA, mostly referred to as VEGF, is a member of PDGF superfamily together with other four proteins with structural homology to VEGF; VEGF-B, VEGF-C, VEGF-D, VEGF-E (Neufeld et al., 1999). VEGFA ligand is largely expressed in vascular endothelial cells and a variety of non-endothelial cells such as macrophages and monocytes. VEGF is capable to bind primarily to vascular growth factor receptor-1 (VEGFR-1) with higher affinity than VEGFR-2 thus mediating cell survival, proliferation and angiogenesis via activation of both VEGFR-1 and VEGFR-2 (Takahashi and Shibuya, 2005). The integrins are a large family of cell adhesion receptors which mediate cell to cell and cell to extracellular matrix interactions. The integrins play a significant role in glioma cell invasion and are considered to be potential therapeutic targets (D'Abaco and Kaye, 2007).

As yet, no single therapy including EGFR inhibitor erlotinib (van den Bent et al., 2009b) and gefitinib (Rich et al., 2004), Protein Kinase C (PKC) / phosphoinositol-3-kinase (PI3K/Akt) inhibitor enzastaurin (Wick et al., 2010) and platelet derived growth factor receptor-alpha (PRGFR) / mast cell/stem cell growth factor (cKit) / break point cluster region-abelson (Bcr-Abl) inhibitor imatinib (Wen et al., 2006a) has been successful in demonstrating a significant survival advantage compared with standard therapy regimens. Recently, a multi-kinase angiogenesis inhibitor drug, Pazopanib, targeting vascular endothelial growth factor receptor-1, -2 and -3 (VEGFR-1, -2 and -3), PDGFR- α , PDGFR- β and c-KIT has also demonstrated no clinically significant improvement in PFS6 (Iwamoto et al., 2010). However, in a phase I/IIa trial, an angiogenesis inhibitor and $\alpha\beta3/5$ integrin antagonist cilengitide (500mg twice in a week) administered with concurrent TMZ has been shown to provide a survival advantage for the newly diagnosed glioblastoma patients with *MGMT* promoter

methylation (Stupp et al., 2010c). This pioneering study has led to the development of two additional clinical trials, CENTRIC and ExCentric. Whilst the recently completed (in June, 2011) phase III trial CENTRIC, designed for efficacy and safety testing of cilengitide (2000mg twice in a week), recruited only newly diagnosed patients with methylated *MGMT* promoter (Stupp et al., 2010b), the phase II ExCentric trial (ongoing) assesses the efficacy of intense dose cilengitide (2000mg twice in a week) combined with low dose procarbazine in addition to concurrent radiotherapy and TMZ treatment in newly diagnosed glioblastoma patients with unmethylated *MGMT* promoter (Khasraw et al., 2011). In addition to cilengitide, another angiogenesis inhibitor, bevacizumab (VEGF antagonist) has been suggested to demonstrate modest survival benefit in glioblastoma patients. In 2007, Vredenburgh and colleagues conducted the first phase II trial of bevacizumab combined with topoisomerase I inhibitor, irinotecan which resulted in slightly improved median PFS of 5 months compared to previous studies conducted with anti-angiogenic therapies in recurrent glioblastoma patients (Vredenburgh et al., 2007).

There are a few mechanisms which are proposed to be involved in mechanism of action for bevacizumab; a direct inhibitory action on VEGFR-expressing glioma cells, disruption of the microvascular niche of glioma stem cells and vascular normalisation by improving dysfunctional vascular physiology of glioma and increasing intratumoral oxygenation (Chamberlain, 2011). However, an issue was revealed for bevacizumab therapy regarding its anti-permeabilisation function which gives rise to a temporary radiographically reduced contrast enhancing image of the tumour area during the course of bevacizumab therapy and therefore treatment regimens including bevacizumab can result in occurrence of so-called 'pseudo-response' (Chamberlain, 2011). More recently, In a phase II bevacizumab trial including seventy newly diagnosed glioma patients

treated with concurrent bevacizumab with TMZ and the researchers reported that the overall survival of 19.6 months was improved compared to the OS of 14.6 months from European Organisation for Research and Treatment of Cancer-National Cancer Institute of Canada cohort while no improvement was observed compared to OS of 21.1 months previously obtained from University of California, Los Angeles/KPLA control cohort (Lai et al., 2011). Additional studies are required to better understand mechanism of action and clinical value of bevacizumab treatment.

A number of examples of other novel targeted therapies included vaccination therapies, radioimmunotherapy and NovoTTF. Regarding vaccination therapies based on autologous mature dendritic cells pulsed with glioblastoma specific peptide or whole tumour lysates, EGFRvIII-peptide vaccination has shown some promising results in newly diagnosed glioblastoma patients (Sampson et al., 2009; Sampson et al., 2010). However, this tumour specific EGFR variant is expressed only in a small subset of glioblastomas and findings of phase II trial is needed to be investigated further in a randomised phase III trial (Sampson et al., 2009; Sampson et al., 2010). Radioimmunotherapy is another potentially encouraging approach examined in treatment of glioblastoma which utilises radiolabeled monoclonal antibodies raised against EGFR, tenascin and integrins in preclinical and phase II trials (Buonerba et al., 2011; Li et al., 2010; Veeravagu et al., 2008). Moreover, recent FDA approved device, NovoTTF employing electrical tumour treatment fields has been suggested to be an innovative alternative treatment to the best available chemotherapy as salvage therapy in recurrent patients. Although the overall survival advantage is non-significant, median overall survival reported for NovoTTF group and chemotherapy group was 6.6 versus 6.0 months, respectively. A phase III trial evaluating efficacy of NovoTTF together with temozolomide versus temozolomide alone in newly diagnosed glioblastoma patients is

currently in progress (Stupp and Weller, 2010; Stupp et al., 2010a). Combination of these advanced modalities with more effective therapies targeting multiple signalling pathways which involve cellular proliferation, migration, invasion and angiogenesis of tumour and its microenvironment may further improve survival of glioma patients in future.

1.2 TOPOISOMERASE II ALPHA, A POTENTIAL TARGET FOR ETOPOSIDE

1.2.1 Topoisomerases

Topoisomerases were first recognised with the discovery of Topoisomerase I in *Escherichia coli* by James Wang who characterized the ability of Topoisomerase to remove negative supercoiling from bacterial DNA (Wang, 1971). Topoisomerases are highly conserved nuclear enzymes which are responsible for the control and modulation of the topological states of DNA. They are classified into two subgroups which are known as topoisomerase I and II. These two isoforms are expressed by different genes. While Type I Topoisomerases (TopoI) have a basic function in transcription, Type II Topoisomerases (TopoII) perform important roles in DNA replication and mitotic events (Kellner et al., 1997). TopoII enzymes are responsible for the segregation of newly replicated chromosome pairs in the condensation and de-condensation of chromosome (Berger et al., 1996).

1.2.1.1 Structures of Topoisomerase II alpha (TopoII α) and beta (TopoII β)

It has been postulated that the 180-kDa β -isozyme may function in the transcriptional regulation of ribosomal genes (Zini et al., 1992). Expression level of this isoform has been found to be independent of the cell cycle. In contrast, a 170-kDa α -isoform demonstrated fluctuating activity resulting in variable expression levels. The function of TopoII α is strongly related to the cell proliferation (Woessner et al., 1991). TopoII α and

β have very similar primary structures which are divided into three domains. The N-terminal domain of TopoII contains ATP binding and hydrolysis sites. An active site tyrosine responsible for DNA cleavage and ligation is located in the central domain of TopoII. The C-terminal domain is remarkably variable between the two human isoforms. This portion of the enzyme contains nuclear localisation sequences and phosphorylation sites (see Figure 1.3). In recent studies, it has been indicated that C-terminal domain has a unique ability to supercoil DNA or functions in front of replication forks (Champoux, 2001; McClendon et al., 2007).

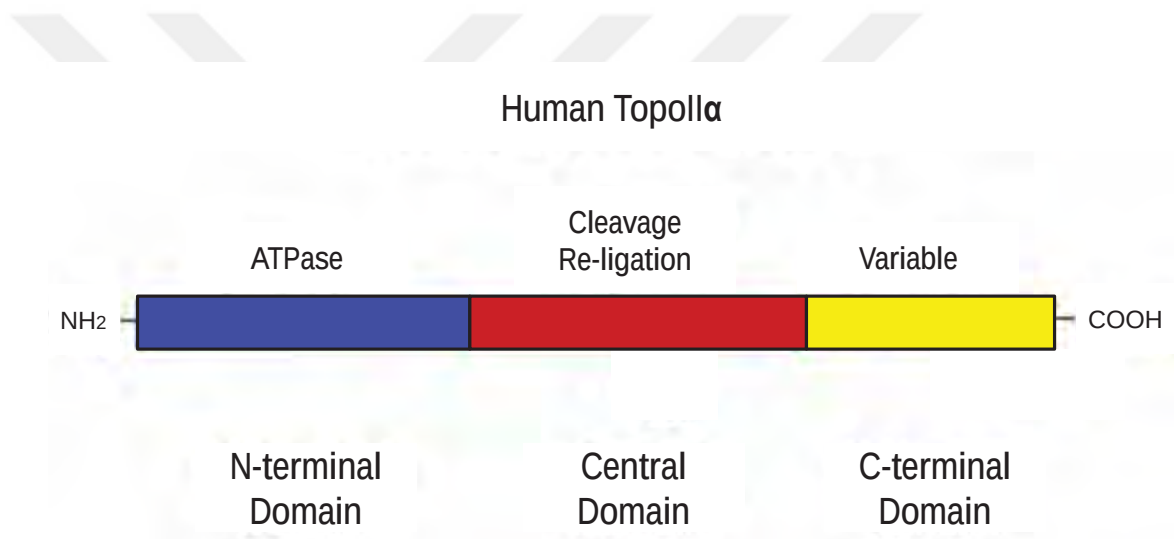


Figure 1.3 Structure of TopoII α

This figure was adapted from ‘DNA topoisomerase II, genotoxicity, and cancer’ (McClendon et al., 2007). Domain structure of human TopoII α is demonstrating N-terminal (blue), central domain (red) and C-terminal (yellow). The N-terminal contains the ATP binding and hydrolysis sites and the central domain is responsible for DNA cleavage and ligation reactions by active tyrosine site. C-terminal domain functions in recognising DNA geometry.

1.2.1.2 Catalytic Cycle of TopoII α

DNA cleaving and ligating are two essential catalytic functions of the TopoII α enzyme. Active tyrosine sites are located in each of the identical subunits of enzyme. Tyrosines are separately responsible for the formation of breaks in one strand of double helix

(Berger et al., 1996). The enzyme forms double cleaved DNA molecules containing four-base linear DNA strand termini. In this cleavage process TopoII α generates covalent bonds with these 5'-terminal phosphate (Zechiedrich et al., 1989). This covalent enzyme-cleaved DNA complex is referred to as the DNA-TopoII α cleavage complex. TopoII α performs its double-stranded passage reaction in the presence of two cofactors, magnesium and adenosine triphosphate (ATP). Magnesium is needed for all steps beyond enzyme-DNA binding as a divalent cation (Osheroff, 1987). ATP is employed in neither DNA cleavage nor re-ligation events; however, the translocation of DNA through the double-stranded nucleic acid gate is performed by ATP. Moreover, hydrolysis of ATP is necessary for enzyme recycling (Baird et al., 1999; Lindsley and Wang, 1991; Lindsley and Wang, 1993).

1.2.1.3 Functions of TopoII α

TopoII α is located on chromosome 17 and this nuclear enzyme is responsible for a cleavage and re-ligation reaction of double strand DNA for the relaxation of torsional stress arising during the replication process (Franceschi et al., 2004; Wilstermann et al., 2007). DNA is underwound (negative supercoiling) in all species and this coiled structure facilitates the separation of complementary DNA strands. Helicases separate the strands of the double helix without unwinding them and the movement of the fork causes a dramatic overwinding (positive supercoiling). Separating the double strand DNA due to the positive supercoils is difficult and it becomes a threatening block to replication, transcription and other crucial DNA processes. These knot and tangle formations accumulate in the genome and terminate the life of the cell due to mitotic failure (see Figure 1.4), (Wang, 2002). There is strong evidence that TopoII α preferentially eliminates positive supercoils that accumulate in front of the replication machinery >10-fold faster than negative supercoils forming behind the replication

machinery as the replication progresses along. In addition, its high affinity to relax positive supercoils results in formation of lower levels of DNA cleavage complexes which decrease the possibility of a collision with the replication machinery causing double-stranded breaks (DSBs). Topoisomerases therefore function to relieve torsional stress ahead of the replication machinery or other DNA tracking systems such as polymerases and helicases (McClendon et al., 2005). Topological state of DNA is modulated through formation of transient DSBs and Topoisomerases remove supercoiling of DNA by passing a double helix through DSBs (Champoux, 2001; Wilstermann et al., 2007). While cleavage complexes are necessary in the catalytic cycle of TopoII α , they are also destructive to the cell. As a consequence, abundance of TopoII α -DNA cleavage complexes in a cell is regulated stringently. DNA-strand breaks initiate multiple recombination/repair pathways. These breaks which accumulate in sufficient concentrations destabilise the genome and programmed cell death pathways are triggered (see Figure 1.3) (Fortune and Osheroff, 2000; Wilstermann and Osheroff, 2003; Wilstermann et al., 2007).

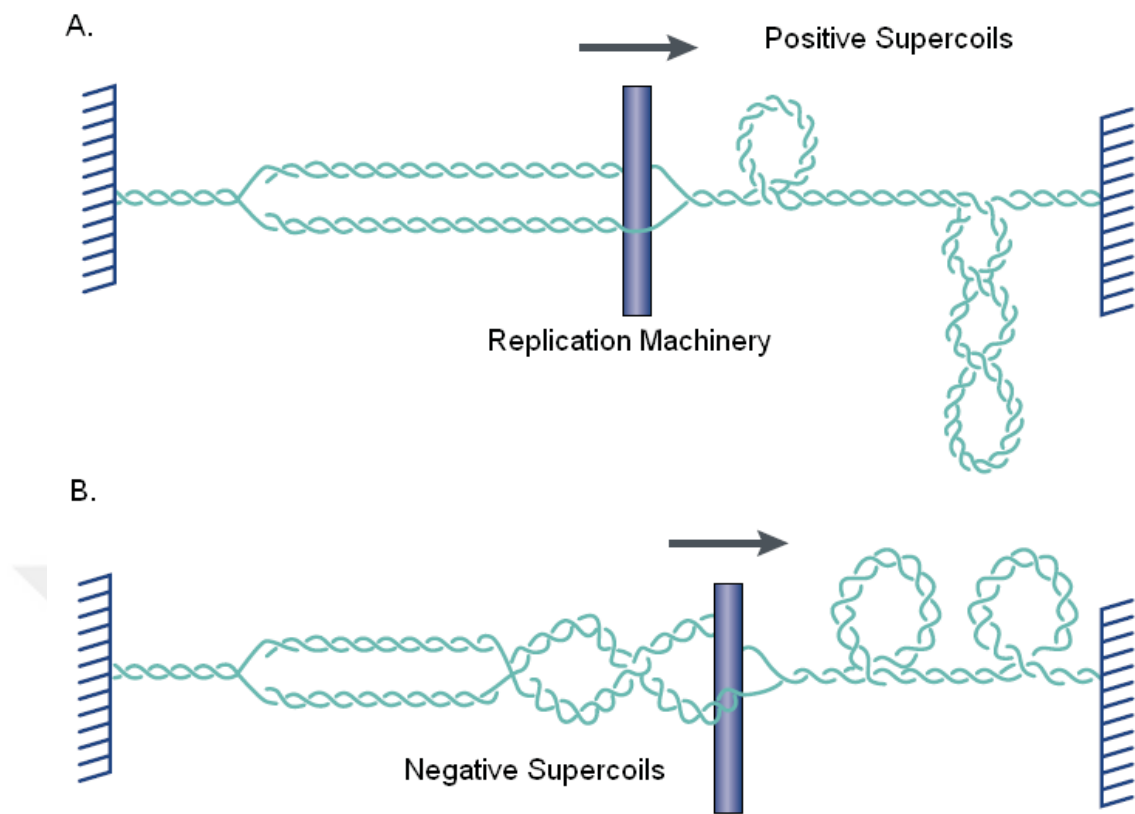


Figure 1.4 Torsional stress and formation of positive and negative DNA supercoils

This figure was adapted from 'Cellular Roles of DNA Topoisomerases: A Molecular Perspective' (Wang, 2002). **(A)** DNA forms positive supercoils ahead of the replication machinery as it passed through the machinery. **(B)** Rotation of replisome around helical axis of DNA produces negative supercoils behind the replication machinery.

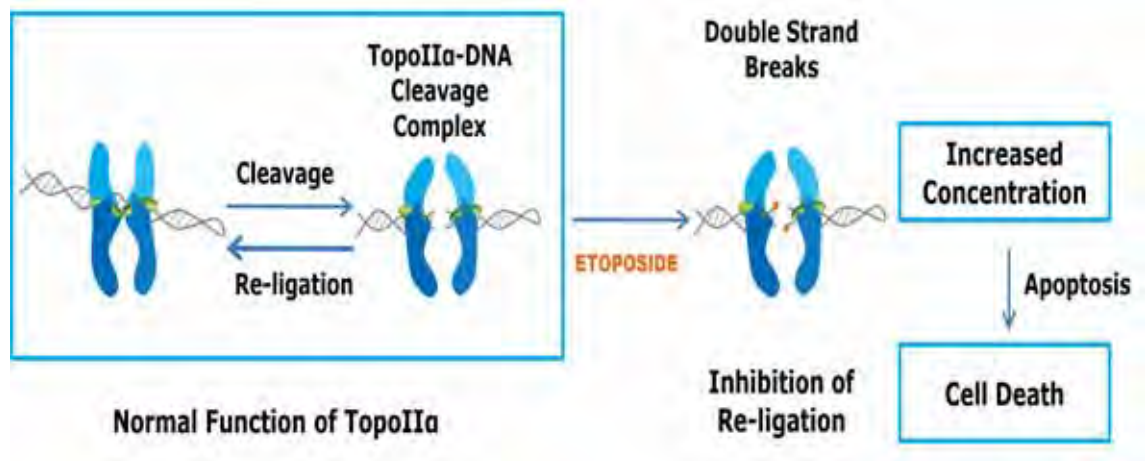


Figure 1.5 Formation of TopoII α -DNA cleavage complex and drug induced DSBs

TopoII α functions in relaxation of DNA supercoils by generating TopoII α -DNA cleavage complexes. Levels of cleavage complexes increase in the presence of etoposide or other TopoII inhibitors. Blockage of re-ligation activity of TopoII α results in DSBs which trigger apoptotic pathways and lead cell death.

1.2.2 TopoII α and Anticancer Drug Interaction

The cellular function of TopoII α as a molecular target for anti-neoplastic agents is not entirely known. Drugs which manipulate potentially lethal function of TopoII α are referred as TopoII α inhibitors and are included in nearly half of the current chemotherapeutic regimens (Bromberg et al., 2004). In other words, TopoII α enzyme is a cytotoxic target for a number of anticancer drugs such as amsacrine, doxorubicin, genistein and mitoxantrone as well as etoposide (McClendon et al., 2007). Anticancer drugs that specifically target TopoII α increase cellular levels of the cleavage complexes that are formed during the scission event (Deweese et al., 2008). Suppression of TopoII α in HeLa cells by RNA interference has shown that the inhibition of TopoII α leads to the resistance to drugs that poison this enzyme (Gudkov et al., 1993b). TopoII α -targeted anti-neoplastic agents inhibit the re-ligation step by binding to TopoII α -DNA

complexes. Thus, DNA DSBs are converted to stable defects which are able to trigger apoptosis (Bromberg and Osheroff, 2001; MacGrogan et al., 2003). Decreased generation of drug-stabilized cleavable TopoII-DNA complexes is observed in quiescent cells expressing low levels of TopoII and this results in less breaks in the genome. Correspondingly, low levels of TopoII results in reduction in the signals of apoptosis in cells (Asano et al., 1996). In addition, mutations in the TopoII α gene can also lead to drug resistance (Vassetzky et al., 1995). Transfection of the human TopoII α gene into a brain tumour cell line resulted in increased expression and sensitivity to etoposide have observed (Asano et al., 1996).

1.2.2.1 Etoposide as an Anticancer Drug

Etoposide is a highly potent anticancer drug that has been used for the treatment of human cancers for over 20 years. It is employed as a therapy for a variety of malignancies, including leukemias, lymphomas, and several solid tumours (Bromberg et al., 2003; Wilstermann et al., 2007). It is also known as VP-16 which is a semi-synthetic derivative of epipodophyllotoxin and exerts its function by directly interacting with TopoII α to cause damage on the DNA (Fulton et al., 1996b). A number of studies revealed that etoposide can penetrate glioma by either moving through the normal blood-brain barrier or transporting across a defective blood-brain barrier in malignant tumours (Fulton et al., 1996b; Kiya et al., 1992; Stewart et al., 1984). Fulton and co-workers used prolonged low dose oral etoposide to result in a 20% response rate in patients with recurrent glioma (Fulton et al., 1996a). Etoposide is most commonly used in combination with carboplatin due to synergistic interactions between etoposide and platinum-based agents (Stein et al., 1999). Application of this regimen as a second line therapy following failure of post-operative radiation and chemotherapy has shown slight efficacy and good tolerability in recurrent patients (Watanabe et al., 2002).

1.2.3 Association between *p53*, *TopoII α* and response to Etoposide Treatment

Transcriptional regulation of *TopoII α* by wild type *p53* is achieved by the binding of *p53* to the specific sites in the *TopoII α* promoter (Wang et al., 1997a). While wild-type *p53* suppresses transcription of *TopoII α* by targeting specific binding sequences for promoter activity, mutant *p53* is not capable of diminishing the activity of the *TopoII α* gene promoter (Sandri et al., 1996). It has also been stated that cell proliferation, differentiation and drug sensitivity in tumour cell lines may be affected by the mutations of the gene encoding for *TopoII α* . Accordingly, *TopoII α* can be used as a marker for cell proliferation and thus, a marker of poor prognosis (Kellner et al., 1997).

Studies have shown that *TopoII α* -mediated DNA breakage can increase from the inhibiting effects of Etoposide in the re-ligation step of nucleic acids. Etoposide kills cells in any stage of the cell cycle, however higher sensitivity has been indicated from mid-S to G2 phase. A study revealed that 2- to 3-fold more sensitivity to etoposide was observed in cells from mid/late S-phase to G2 than cells in G1. Accordingly, it can be stated that chromosomal problems arising during DNA synthesis in the presence of etoposide are more effective to initiate apoptotic pathways (Montecucco et al., 2007). Etoposide not only induces formation of *TopoII α* cleavable complexes but also activates various DNA damage molecules, such as Ataxia Telangiectasia mutated protein (ATM), Histone 2AX (H2AX), Checkpoint kinase 1 and 2 (Chk1/2), *p53*, and replication protein A (RPA). Moreover, etoposide gives rise to cellular responses involving cell cycle arrest, non-homologous end joining, homologous recombination repair and cell death (Fan et al., 2008).

Only a handful of studies investigated the degradation mechanisms of Topoisomerases in the last two decades (Desai et al., 1997; Mao et al., 2000; Mao et al., 2001; Xiao et

al., 2003) . TopoI-cleavage complexes have been mostly studied and ubiquitin was reported to initiate degradation of TopoI-cleavage complexes via 26S proteasome pathway, resulting in down-regulated levels of TopoI enzyme following treatment with the TopoI inhibitor drug, Camptothecin (Desai et al., 1997). Moreover, stimulating effects of Camptothecin in conjugation of small ubiquitin-like modifier-1 (SUMO-1) to TopoI has also been shown (Mao et al., 2000). Remarkably, degradation of Topoisomerases possibly results in dissociation of cleavage complexes which presumably unveil bare DSBs and in turn initiate DNA repair mechanisms (Pouliot et al., 1999). A repair mechanism has been suggested in relation to phosphodiester bonds formed between tyrosine residue of TopoII and 5' end of the DNA break. A repair enzyme, tyrosyl-DNA phosphodiesterase 1 (TDP1), has been explored to play a role in breaking these covalent bonds located in drug-stabilised cleavage complex core (Pouliot et al., 2001; Pouliot et al., 1999). In the light of these findings, more recently overexpression of TDP1 has been shown to significantly reduce etoposide-initiated DNA damage (Barthelmes et al., 2004).

1.2.4 TopoII α , Solid Cancers and Glioma

Expression levels of TopoII α protein have been associated with response to TopoII α inhibitors in preclinical *in vitro* studies. (Gudkov et al., 1993a; Jarvinen et al., 2000). Correlation of TopoII α expression with human epidermal receptor 2 (HER2) expression levels have been comprehensively studied in breast cancer. *TopoII α* gene is located at the same chromosomal locus (17q12-q21) as *HER2* gene and TopoII α is also co-expressed (Oakman et al., 2009). In breast cancer, 57% of *HER2* amplified tumours have been reported to show aberrations in *TopoII α* gene. Prevalence of *TopoII α* gene aberrations have also found in 5-10% of gastric, lung and bladder tumours (Mano et al., 2007). Increased responsiveness of *HER2*-amplified breast cancers to a TopoII α

inhibitor, doxorubicin has also been reported (Muss et al., 1994). Despite the extensive research into TopoII α and HER2 in breast cancer, very little is known about its role in brain tumours.

In the late 90s, Asano and co-workers transfected TopoII α into a resistant glioma cell line in order to increase its sensitivity to etoposide, and achieved a transient sensitivity (Asano et al., 1996). Expression of TopoII α has been observed at varying frequency by 11-36% of high-grade gliomas, 4% of low-grade gliomas and 7% of medulloblastoma in tumour samples (Franceschi et al., 2004). TopoII α expression levels in high-grade glioma cells indicate several criteria, such as the responsiveness to chemotherapy, proliferative activity of these cells and mutations in the enzyme which is caused by malignant transformation (Bredel et al., 2002a). Variations in the activity of TopoII α have important roles in the formation of a resistant phenotype in a group of intracranial neoplasms. Consequently, both high and low expressions of TopoII α in patients with glioma provide predictive outcome with a beneficial prognosis (Bredel et al., 2002a).

1.3 NG2 PROTEOGLYCAN

The extracellular matrix (ECM) consists of numerous proteins, collagens, proteoglycans and polysaccharides that contribute to regulation of a multifaceted signalling network (Lukashev and Werb, 1998). The ECM of the central nervous system (CNS) is an environment rich in fibronectin, collagens laminins, hyaluronan, tenascins, link proteins and proteoglycans; predominantly chondroitin sulfate proteoglycans (CSPG) (Zimmermann and Dours-Zimmermann, 2008). Under normal conditions, specialised functions of the ECM components in the CNS involve developmental processes, maintenance of CNS ionic homeostasis and providing physical support for neurons and glial cells (Bandtlow and Zimmermann, 2000). However, variations in the expression

levels of these elements are often reported in pathological states such as spinal cord injuries (Jones et al., 2003; Sandvig et al., 2004), inflammatory de-myelinating diseases (eg. multiple sclerosis) (Sobel and Ahmed, 2001), and gliomas (Bauer et al., 2011; Chintala et al., 1996; Mahesparan et al., 2003). Proteoglycans contain glycosaminoglycan (GAG) chains that covalently linked to a core protein. Proteoglycans are classified into subgroups such as aggrecan, brevican, versican, neurocan, phosphocan and neuronal/glia 2 (NG2) depending on the type of their core proteins (Haylock-Jacobs et al., 2011).

1.3.1 Neuronal/glia 2(NG2)

NG2 (also termed CSPG4 and melanoma-associated proteoglycan) is a membrane-spanning proteoglycan that contributes to signal transduction via growth factor receptors and $\beta 1$ integrins (Stallcup and Huang, 2008). The NG2 core protein is divided into three sub-complexes including a transmembrane domain (25 aa), cytoplasmic domain (76 aa) and a large extracellular domain (2225 aa) (Nishiyama et al., 1991b). Domains of this single-pass membrane protein play a pivotal role in the functional interaction between intracellular and extracellular elements. The NG2 extracellular domain contains a number of specific sites for the binding of collagen type V and VI and several other extracellular matrix molecules such as laminin-1 and tenascin-C (Subdomain-2) (Burg et al., 1996; Tillet et al., 1997). The extracellular domain also contains intramolecular disulfide bonds (Subdomain-1), a single chondroitin sulfate chain at ser-999.14 (Subdomain-2), N-linked oligosaccharides that are required for galectin-3 binding (Subdomain-3) and sites for proteolytic processing of NG2 (Subdomain-3) (Nishiyama et al., 1995; Trotter et al., 2010; Wen et al., 2006b). The cytoplasmic domain of NG2 comprises a number cytoplasmic threonine phosphorylation sites. Phosphorylation at the sites Thr-2256 and Thr-2314 has been

associated with some important functional implications such as cell polarisation and motility, cell proliferation and migration, respectively (Makagiansar et al., 2007; Makagiansar et al., 2004). The cytoplasmic tail of the protein also contains a proline-rich region. However, this region does not exactly match Src homology 3 (SH3)-binding motifs (PXXP) (Fang et al., 1999). In addition, QYWV sequence of the PDZ-binding motif which is responsible for the binding of scaffolding proteins, multi PDZ domain protein 1 (MUPP1), glutamate receptor interacting protein (GRIP1) and syntenin-1 exists within the cytoplasmic domain (Barritt et al., 2000; Chatterjee et al., 2008; Songyang et al., 1997; Stegmuller et al., 2003)

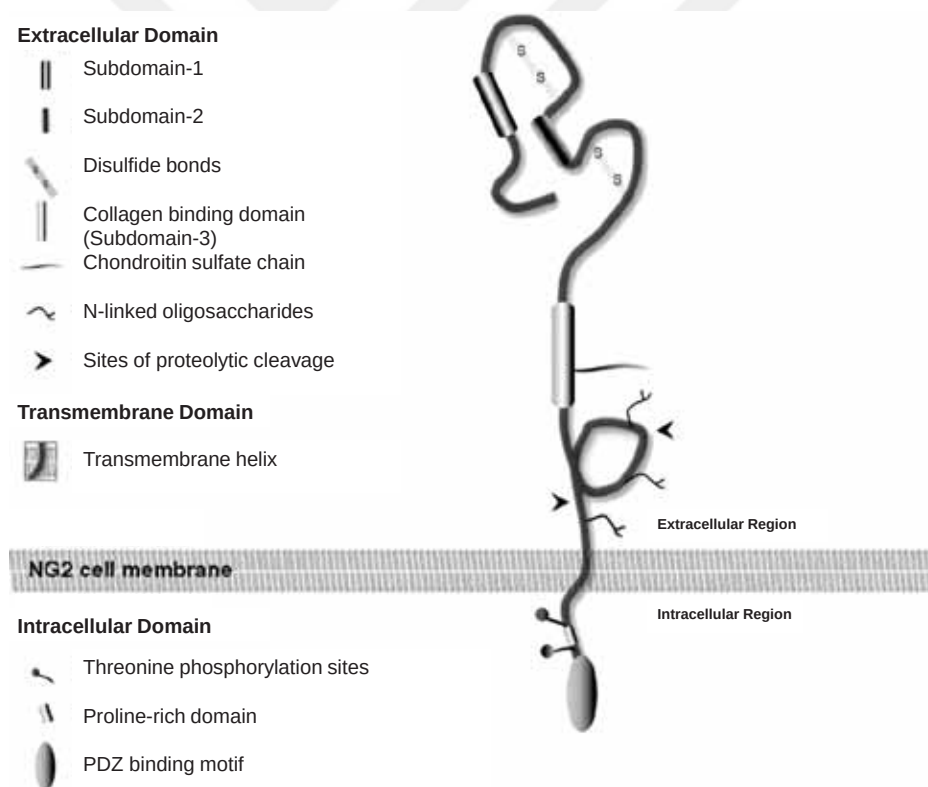


Figure 1.6 The schematic structure of NG2

This figure was adapted from ‘NG2 cells: properties, progeny and origin’ (Trotter et al., 2010). The extracellular domain of NG2 comprises three subdomains functioning in the ligand binding such as collagen type V and VI, laminin-1 and tenascin-C. The intracellular domain contains several sites for phosphorylation (Threonine 2256, Thr-2314), PDZ-binding motifs and proline-rich regions.

1.3.2 Expression of NG2 in Different Cell Types

NG2 is expressed by several different progenitor cell types such as immature chondroblasts (Fukushi et al., 2003; Nishiyama et al., 1991a), osteoblasts (Fukushi et al., 2003), immature keratinocytes (Kadoya et al., 2008) and oligodendrocyte progenitor cells (OPCs). Vascular smooth muscle cells also express NG2 which result in elevated signalling via PDGFA and increased response to growth factors in normal growth or pathological conditions including atherosclerosis and artery injuries (Grako and Stallcup, 1995). Expression of NG2 is also detected in microvasculature pericytes during angiogenesis of tumour or development of normal vasculature (Burg et al., 1999; Ozerdem et al., 2002; Ozerdem et al., 2001; Schlingemann et al., 1990). Since the excessive vascularisation is a hallmark of glioma (Burger and Kleihues, 1989; Plate and Risau, 1995), NG2 expression in glioma vasculature has been investigated and intense expression of NG2 has been reported by a number of different research groups (Chekenya et al., 2002b; Schlingemann et al., 1990; Shoshan et al., 1999; Wesseling et al., 1995).

NG2 expression in central nervous system is mainly limited to the OPCs which are also characterised by co-expression of PDGFRA (Chang et al., 2000). Terminal differentiation of NG2-expressing OPCs to oligodendrocytes results in reduced levels of NG2 expression (Stallcup and Beasley, 1987). There is some evidence suggesting that OPCs originate from NG2-positive pre-OPCs (Baracska et al., 2007). Among glial progenitors, NG2-expressing oligodendrocyte progenitor lineage-derived cells have been suggested to represent a cellular origin for progression of oligodendrogliomas (Briancon-Marjollet et al., 2010; Persson et al., 2010). The potential role of NG2-expressing OPCs as cells of origin for gliomas is extensively discussed in Section 1.1.2, Literature Review, Chapter 1.

1.3.3 Functions of NG2

NG2 appears to play important roles in potentiating invasive and metastatic potential of the cells in malignancies such as glioma and melanoma (Burg et al., 1998; Chekenya et al., 2002a). Expression of NG2 is widely documented in various types of cancers including glioblastomas (Schrappe et al., 1991; Shoshan et al., 1999; Staugaitis and Trapp, 2009), acute myeloid leukemia (Behm et al., 1996), chondrosarcoma (Leger et al., 1994), soft-tissue sarcoma (Benassi et al., 2009) and melanomas (Price et al., 2011; Schlingemann et al., 1990). NG2 has been suggested to function in the neoangiogenic processes and it has been shown that sequestration of angiostatin by NG2 supports growth of new blood vessels and pericytes maintaining angiogenic sprouts and tube formation (Chekenya et al., 2002a; Goretzki et al., 2000; Ozerdem and Stallcup, 2003).

NG2 elicits a critical function in facilitating cell motility (Fang et al., 1999) and mediating cellular responses to growth factor signalling (Goretzki et al., 1999; Grako and Stallcup, 1995). Fukushi and colleagues showed that the use of soluble recombinant NG2 both *in vitro* and *in vivo* experimental models stimulated motility in vascular endothelial cells (Fukushi et al., 2004). In this study, the interaction of NG2 with the galectin-3 and $\alpha 3\beta 1$ integrin complex was demonstrated to be strongly associated with increased cell motility, enhanced endothelial cell motility and attributed to augmented formation of blood vessels *in vivo* (Fukushi et al., 2004). The functional role of NG2 co-localised with a $\alpha 3\beta 1$ integrin complex has been investigated in U251 human astrocytoma cells which predominantly express $\alpha 3\beta 1$ integrin but low levels of NG2. U251 and NG2-transfected U251 cells indicated no difference in their motilities, whereas stimulation of the cells with phorbol 12-myristate 13-acetate (PMA) [an activator of Protein Kinase C (PKC)], and PDGF increased cell motility considerably (Makagiansar et al., 2004). The same researchers demonstrated that PMA and PDGF-

dependent phosphorylation of NG2 at Thr-2256 resulted in enhanced motility, while increased cell proliferation has been reported to be a consequence of ERK-induced phosphorylation of NG2 at Thr-2314 (Makagiansar et al., 2007; Makagiansar et al., 2004). They also reported that co-localisation of Thr-2314-phosphorylated NG2 with $\alpha 3\beta 1$ on microprotrusions on the apical cell surface increases proliferation of the cells through direct interaction with various ligands, however, phosphorylation of NG2 at Thr 2256 co-localised with $\alpha 3\beta 1$ along lamellipodial edges of the cell instigated increased motility by interacting elements of cytoplasm (Makagiansar et al., 2007; Makagiansar et al., 2004). NG2 has also been shown to serve as a co-receptor of $\alpha 4\beta 1$ and activation of both give rise to elevated signalling in downstream effectors such as focal adhesion kinase (FAK) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) which are known to mediate migration, tumour growth and survival (Eisenmann et al., 1999; Iida et al., 1995; Yang et al., 2004a). Therefore, the diversity of signal molecules phosphorylating NG2 at different Thr residues can lead different functional processes that determine the cell fate. A number of molecules such as GRIP1 (Stegmuller et al., 2003), syntenin-1 (Chatterjee et al., 2008), MUPP1 (Barritt et al., 2000) and ezrin (Makagiansar et al., 2004) have been identified to be the possible ligands that convey their information by interacting with NG2 and integrin complexes. In addition to these, NG2 displays increased affinity to growth factors, fibroblast growth factor-2 (FGF-2) and Human platelet derived growth factor AA (PDGF-AA), and binds them via the core protein which subsequently promote cell proliferation affinity (Cattaruzza et al., 2008; Goretzki et al., 1999).

Signal transduction occurring between ECM components (Collagens V and VI, fibronectin and laminin) and NG2 has important functions in cell proliferation and motility (Iida et al., 1995; Tillett et al., 2002). Cell motility can be modulated by the

attachment of PDZ-domain binding molecule MUPP1 to NG2 which in turn triggers cytoskeletal re-organisation through phosphorylation of FAK and activation of downstream Ras-related C3 botulinum toxin substrate 1 (rac1) and cell division control protein 42 (cdc42) that regulate actin microfilament and microtubular networks in the migrating cell (Majumdar et al., 2003). Interaction of NG2 with matrix metalloproteinases (MMP) and specifically membrane-type 3 MMP (MT3-MMP) have been shown to contribute to the matrix-degrading ability of melanoma cells (Iida et al., 2001). In this study, co-immunoprecipitation results also demonstrated that MT3-MMP act entirely independent of $\alpha\text{v}\beta 3$ integrin, however, a moderate level of interaction has been indicated regarding its relationship with $\beta 1$ integrin complexes (Iida et al., 2001).

NG2 also plays a vital role in cell survival as well as cell proliferation and migration of glioma cells. NG2 controls cell survival through activation of $\alpha 3\beta 1$ integrin in a NG2-dependent manner and enhances survival signalling via PI3K/Akt pathway (Chekenya et al., 2008). Contribution of NG2 to cell survival is an important factor for gliomas characterised with chemoresistance to numerous treatments with chemotherapeutic agents (Chekenya et al., 2008). Chekenya *et al.* demonstrated that U251 cells transfected with NG2 represented high level of resistance to treatment including TNF α and chemotherapeutic agents; doxorubicin, vincristine and etoposide while therapy responsiveness and increased apoptosis were reported in untransfected U251 cells (Chekenya et al., 2008). In this study, the researchers also conducted knockdown experiments using NG2-transfected U251 and showed that siRNA suppressed NG2 expression re-established chemotherapy sensitivity of the cells (Chekenya et al., 2008).

1.3.4 NG2 Expression in Glioma

NG2 overexpression has been reported in 50% of the tumours from newly diagnosed glioblastoma patients (n = 74) and significantly correlated with poor survival (Svendsen et al., 2011). In this study, patients with low NG2 expression levels, a survival benefit of 4.5 months was measured compared to the patients expressing significantly higher levels of NG2 (OS = 8 months) (Svendsen et al., 2011). Al-Mayhani and colleagues provided some evidence that NG2-positive glioma cells had higher proliferation and tumorigenic potential than NG2-negative cells (Al-Mayhani et al., 2011). They also reported upregulated expression of several transcription factors such as maternal embryonic leucine zipper kinase (MELK), cell division cycle (CDC), micro-chromosome maintenance (MCM) and E2 promoter binding factor (E2F) genes associated with shorter overall survival in a subset of glioma cells expressing NG2 (Al-Mayhani et al., 2011). Aggressive tumour growth and angiogenic activity was reported in nude rat xenografts implanted with NG2-overexpressing glioma cell line (U251-transfected with NG2) in comparison to those rats implanted with the non-transfected U251 cells (Wang et al., 2011a). Moreover, treatment of xenografted tumours with lentiviral vectors encoding NG2 shRNAs resulted in reduction in tumour growth, angiogenesis, oedema levels and normalised tumour vascularity (Wang et al., 2011a). Involvement of NG2 in multiple pathways associated with cell motility, proliferation, survival, angiogenesis and chemoresistance indicate its diverse functional potential and can be critical for glioma progression and tumour cell resistance (Stallcup and Huang, 2008).

1.3.5 Cilengitide

Cilengitide [EMD 121974] is a peptide-like molecule containing an RGD sequence that binds to $\alpha v\beta 1$ and $\alpha v\beta 5$ integrins with high affinity and blocks their signalling to downstream targets (Smith, 2003). RGD sequences are also recognised by numerous integrin complexes including $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha I\text{Ib}\beta 3$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 7\beta 1$ (Ruoslahti, 1996). In an animal glioma model, prolonged survival times have been shown in mice treated with cilengitide compared to inactive control peptide-treated group (MacDonald et al., 2001). A randomised phase II study reported that the effective delivery of cilengitide to glioblastoma with no dose-limiting toxicities. In addition, a median survival of 9.9 months and a moderate level of antitumor activity were recorded in recurrent glioma patients treated with 2000 mg of cilengitide twice weekly for four weeks (Reardon et al., 2008). Recently, the results of a North American Brain Tumor Consortium (NABTC) study confirmed the improved delivery efficacy of cilengitide and its modest activity when used as a single therapeutic agent in recurrent gliomas (Gilbert et al., 2012). In an *in vitro* study, cilengitide combined with TMZ has been demonstrated to induce cell detachment and apoptosis in MGMT promoter methylated glioma cells (Oliveira-Ferrer et al., 2008). However, a preclinical study using integrin inhibitors S 36578 and cilengitide in an animal model with Lewis lung carcinoma (LLC) and melanoma showed a controversial effect of these inhibitors at nanomolar concentrations whereby tumour growth and angiogenesis was stimulated while anti-angiogenic effects of the inhibitors was observed at high dose (Reynolds et al., 2009). In addition, Maurer and colleagues reported that differential response of the glioma cells to cilengitide was observed, independent of their expression levels of $\alpha v\beta 3$ and $\alpha v\beta 5$ (Maurer et al., 2009). An ongoing phase III clinical trial, CENTRIC, has been designed to evaluate the efficacy of cilengitide combined with radiotherapy/TMZ in

newly diagnosed glioblastoma patients with methylated MGMT promoter (Stupp et al., 2010b).

1.4 PROTEOMICS

1.4.1 Definition of Proteomics

The term 'proteome' refers to entire complement of proteins encoded and expressed by a genome (Wasinger et al., 1995). Ultimate function of the proteins and the dynamic nature of the proteome is determined by modulations at points from post-transcription (alternative splicing and polyadenylation) to post-translation (phosphorylation, dephosphorylation, glycosylation, cleavage and complex formation) (Banks et al., 2000). These proteome alterations result in expression of, in many cases, modified versions of the same protein encoded by one gene (Banks et al., 2000).

1.4.2 Proteomic Technologies

Proteomic technologies are wide-scale analyses specified to study a single protein to hundreds or thousands of proteins in one experiment (Sheta et al., 2006). Several proteomic techniques involving distinct modes of analysis of proteins are available at present and are detailed in the following sections.

1.4.2.1 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE is the most commonly used technique in proteomics (Carrette et al., 2006). In 2D-PAGE, proteins are separated according to their isoelectric points in the first dimension and their molecular weight in the presence of sodium dodecyl sulphate (SDS) in the second dimension (Issaq and Veenstra, 2008). This method compares

protein expression pattern between different samples on separate gels. An average of 1,000-2,500 spots corresponding to ~300-1,000 proteins can be generated on a single gel. After the separation, proteins are typically quantified using special softwares following the staining of the gel. Traditional post-staining methods include Coomassie brilliant blue, silver staining or fluorescent dyes such as SyproRuby, Deep Purple or Flamingo Red. However, quantitative variability introduced during preparation of samples, sample loading and stain development can result in gel-to-gel variations between two separated gels (Choe and Lee, 2003). Another disadvantage of 2D-PAGE is its lack of potential for real high-throughput technology (Colantonio and Chan, 2005).

1.4.2.2 Two Dimensional Difference Gel Electrophoresis (2D-DIGE)

2D-DIGE technique is developed to minimise gel-to-gel variation by performing gel electrophoresis of the two groups of samples labelled with different dyes (CyDyes: Cy2, Cy3 and Cy5) on the same gel (Minden et al., 2009). For 2D-DIGE, fluorescent labelled dyes react with lysine residues, thus providing high sensitivity and linear quantitation and enables direct visualisation of 500 to 800 proteins in gel (Nicolou et al., 2010). Differentially expressed proteins in individual spots are excised and can be identified by Mass Spectrometry (MS) techniques (Carrette et al., 2006; Nicolou et al., 2010).

1.4.2.3 Protein Microarrays

In addition to these traditional technologies, recent proteomic investigations are mainly concentrated on the applications of high-throughput methods such as protein-detecting microarrays and MS techniques (MacBeath, 2002). Protein-detecting microarrays require antibodies with high affinity and high specificity to capture molecules in a protein mixture. These arrays can be utilised for the discovery of multiple markers to monitor disease progression. However, investigations might be more effective with a

prior knowledge of the proteins functioning in a particular biological process. Because of the limited number of antibodies that can be employed in this technique, only targeted proteins can be revealed by protein microarrays (Kodadek, 2001; MacBeath, 2002).

1.4.2.4 Isotope Coded Affinity Tag (ICAT) and isobaric Tags for Relative and Absolute Quantification (iTRAQ)

ICAT and iTRAQ techniques are stable-isotope labelling chemistries which use mass spectrometry combined with the separation power of Liquid chromatography (LC) (Gygi et al., 1999; Ross et al., 2004). The ICAT method incorporates peptide ions of two different samples which are labelled at cysteine residues with the isotopically light and heavy reagents, respectively. The labelled proteins are mixed and digested with trypsin before being purified on monomeric avidin column. MS analysis of the purified samples produces peptide doublets in mass spectra due to the mass difference between light and heavy isotopes. Abundance of proteins is directly correlated with the intensities of MS spectra of the peptides (Gygi et al., 1999; Wu et al., 2006). ICAT is used efficiently to resolve complex biological samples (Wu et al., 2006). However, limitation of this method to the peptides with cysteine residue and reduction in the number of peptides analysed by MS due to strong affinity some peptide for the avidin column in the pre-treatment steps can appear to be two main drawbacks of this technique (Imai et al., 2011). Moreover, Molloy and colleagues reported a biased detection manner of ICAT toward acidic ($pI < 7$) and small (< 10 kDa) proteins (Molloy et al., 2005). A relatively new method, iTRAQ, employs a 4-fold or 8-fold multiplex isobaric label sets to quantify protein expression in four or eight different samples, respectively. MS/MS signature ions at m/z 114.1, 115.1, 116.1 and 117.1 are generated by the 4-fold multiplex isobaric tags which form an amide linkage to any amines of

peptides of a digested sample (Choe et al., 2007; Ross et al., 2004). The peak area of the generated ions provides relative abundance of peptides derived from initial protein sample (Latterich et al., 2008). Comparison between initial protein amounts and the number of identified proteins by ICAT and iTRAQ suggested a slightly higher sensitivity of iTRAQ than ICAT (Imai et al., 2011). In addition, loss of peptides during pre-treatment procedures of iTRAQ represents similar limitations as ICAT (Imai et al., 2011).

1.4.2.5 Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) and Electro-spray Ionisation (ESI)

Other techniques used for the expression analysis of proteins are MALDI-TOF MS and Surface Enhanced Laser Desorption Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS). The most widely used ionisation technique, MALDI-TOF MS, was first introduced in the mid 1980's by Karas and colleagues (Berkenkamp et al., 1996; Karas et al., 1985). MALDI-TOF MS requires extensive sample pre-treatment steps including sample purification and fractionation, removal of digestion buffers and matrix application on the analyte (McComb et al., 2007). The samples analysed by MALDI-TOF MS are usually trypsin digested proteins which have undergone 2D-PAGE separation (Aebersold and Goodlett, 2001). Prior to MALDI-TOF MS analysis, the complexity of samples is often reduced using peptide separation techniques such as Reverse Phase-Liquid Chromatography (RP-LC) and High Performance Liquid Chromatography (HPLC) (Issaq, 2001; Peng and Gygi, 2001). In MALDI-TOF MS, digested analyte is mixed with a matrix solution to increase the absorption of energy from ultraviolet (UV) light. The sample mixture is then co-crystallised on spots of a sample plate prior to laser shot (He and Chiu, 2003). Laser energy absorbed by the matrix is transferred to the peptides which cause molecules to

ionise. After ionisation, molecules are accelerated and resolved by their different flight time in a TOF tube to reach the detector then a spectrum with peaks representing singly charged masses of peptides is obtained (He and Chiu, 2003). MALDI-TOF MS allows detection of molecules spanning in a wide mass range exceeding 10 kDa up to 100 kDa (Gatlin-Bunai et al., 2007; Karas and Hillenkamp, 1988; Tanaka et al., 1988). MALDI-TOF MS has become a powerful technique also in the mass fingerprinting analysis of complex biological samples and limitations of this technology in identification of hydrophobic peptides and small molecular weight analytes (< 500 Da) have been addressed in several studies (Gonnet et al., 2003; Guo et al., 2002; Lubec and Afjehi-Sadat, 2007).

ESI is another ionisation technique which is used to analyse peptides and nucleic acids (Cech and Enke, 2000). While MALDI tends to favour detection of basic and aromatic species, ESI basically allows selective identification of hydrophobic peptides (Stapels and Barofsky, 2004). Therefore, complementary use of MALDI and ESI coupled with a variety of mass analysers has been suggested in order to avoid biases in the detection of different classes of amino acids (Getie et al., 2005; Stapels and Barofsky, 2004). Hansen and colleagues reported the tendency for ESI to preferentially identify lower molecular masses, whereas MALDI has been suggested to be more sensitive for fewer but larger peptides (Hansen et al., 2003). In addition to classical MALDI-TOF MS approach, over the past decade, MALDI-TOF MS technology coupled with Imaging Mass Spectrometry (IMS) has also been applied to profile and quantify peptides and proteins directly from mammalian cells and tissue sections (Chaurand and Caprioli, 2002; Chaurand et al., 1999; Thibault et al., 2008). However, direct profiling attempts of complex protein samples using MALDI-TOF MS give rise to a certain degree of matrix and analyte suppression in the ionisation step (McCombie and Knochennuss, 2004).

1.4.2.6 Surface Enhanced Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS)

In 1992, Hutchens and Yip pioneered the concept of SELDI-TOF MS technology which enables protein separation on chromatographic or biochemical surfaces (Hutchens and Yip, 1992; Hutchens and Yip, 1993; Hutchens et al., 1992). Among the different MS techniques used to analyse complex biological samples, SELDI-TOF MS is the most suitable high-throughput method for the investigation of low molecular weight proteins (<20 kDa) without pre-treatment steps prior to analysis (Issaq et al., 2003). In addition to MS techniques, an immunology-based assay, Enzyme-Linked Immunosorbent Assay (ELISA) is also used to detect low molecular weight proteins using antibodies specifically targeting proteins of interest. However, availability of suitable antibodies for low molecular weight proteins limits the use of ELISA for protein profiling in lower mass range (Chen et al., 2005).

SELDI-TOF MS application requires a few microliters of a sample on array surface which is followed by an incubation step and removal of unbound proteins and interfering substances by washing with appropriate buffers (Al-Tarawneh and Bencharit, 2009). SELDI-TOF MS employs 8- or 16-spot ProteinChip arrays containing a solid-phase chromatographic surface with different properties (hydrophobic, hydrophilic, anion exchange, cation exchange and metal affinity) or a biochemically active surface treated with biochemical agents (immobilised antibody, receptor, DNA and enzyme) that interact with unique target proteins (see Figure 1.7) (Issaq et al., 2003; Poon, 2007). Various types of these array surfaces combined with different washing buffers enable detection of distinct protein subsets from the samples and thus protein separation of crude biological samples can be achieved (Seibert et al., 2004).

SELDI can be considered as a variant of MALDI-TOF MS because both techniques employ energy absorbing matrix molecules co-crystallised with peptides and the use of UV lasers to ionise molecules (Vorderwulbecke et al., 2005). Similar to MALDI-TOF MS, ionised peptides are accelerated in an electric field and the output is generated from the different mass to charge (m/z) ratios of ion species (see Figure 1.8) (Vorderwulbecke et al., 2005). However, MALDI-TOF MS and SELDI-TOF MS differ in the construction of sample targets, the design of analysis and the software used in data interpretation (Vorderwulbecke et al., 2005). The three key components of SELDI-TOF MS technology are the ProteinChip arrays, the reader and the software (Issaq et al., 2002). Using data analysis software, protein clusters are produced based on m/z values and intensity variation between detected peptides (Popescu et al., 2010). The versatility of SELDI-TOF MS has led to the discovery of novel biomarkers capable of distinguishing cancer from non-cancer and responsive from non-responsive patient groups and by comparing differences between protein expression profiles obtained from protein samples of test groups (Wright, 2002). SELDI-TOF MS provides an effective and sensitive approach for selective profiling of subsets of proteins from complex biological materials such as serum, blood, plasma, urine, saliva, cell lysates, body fluids, cerebrospinal fluid (CSF) and tissues in a fast and simple process (Kalinina et al., 2011a; Popescu et al., 2010; Vorderwulbecke et al., 2005; Wright, 2002). Despite its utility in discovery of potential biomarkers for various diseases, like other novel technologies SELDI-TOF MS has its limitations. It is unable to produce a good-quality resolution of the proteins with molecular weights of larger than 20 kDa (Poon, 2007). In addition, SELDI-TOF MS technology only provides information about the molecular masses of detected biomarkers, and it is critical to know identities of biomarkers to be able to use them as diagnostic, predictive or prognostic tools (Chen et al., 2005). This is

also a drawback that SELDI shares with the original Mass Spectrometric Immuno Assay (MSIA) and with MALDI imaging technologies (Parker et al., 2010). Combination of SELDI technology with MALDI tandem TOF MS which will potentially provide both an advanced peak resolution and direct on-chip identification of detected biomarkers has been suggested to address this limitation (Poon, 2007).

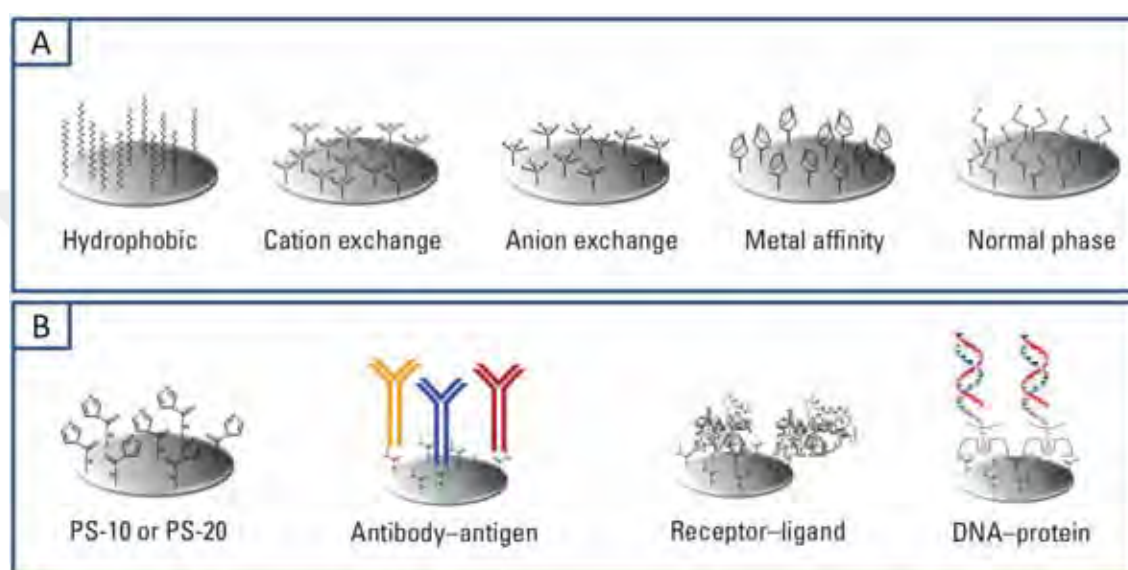


Figure 1.7 SELDI technology utilise various types of modified surfaces to capture and analyse proteins with unique properties

Panel A represents array surfaces which bind proteins according to their physicochemical properties. Specific groups on the surface of biochemically modified arrays capture proteins by reacting with proteins from biological samples, shown in **Panel B**. PS-10 (pre-activated with carbonyldiimidazole) and PS-20 (pre-activated with epoxide chemistry) are both examples of pre-activated biological surfaces and designed to allow non-covalent attachment of amino groups of biomolecules. This figure was adapted from ‘SELDI-TOF MS for Diagnostic Proteomics’ (Issaq et al., 2003).

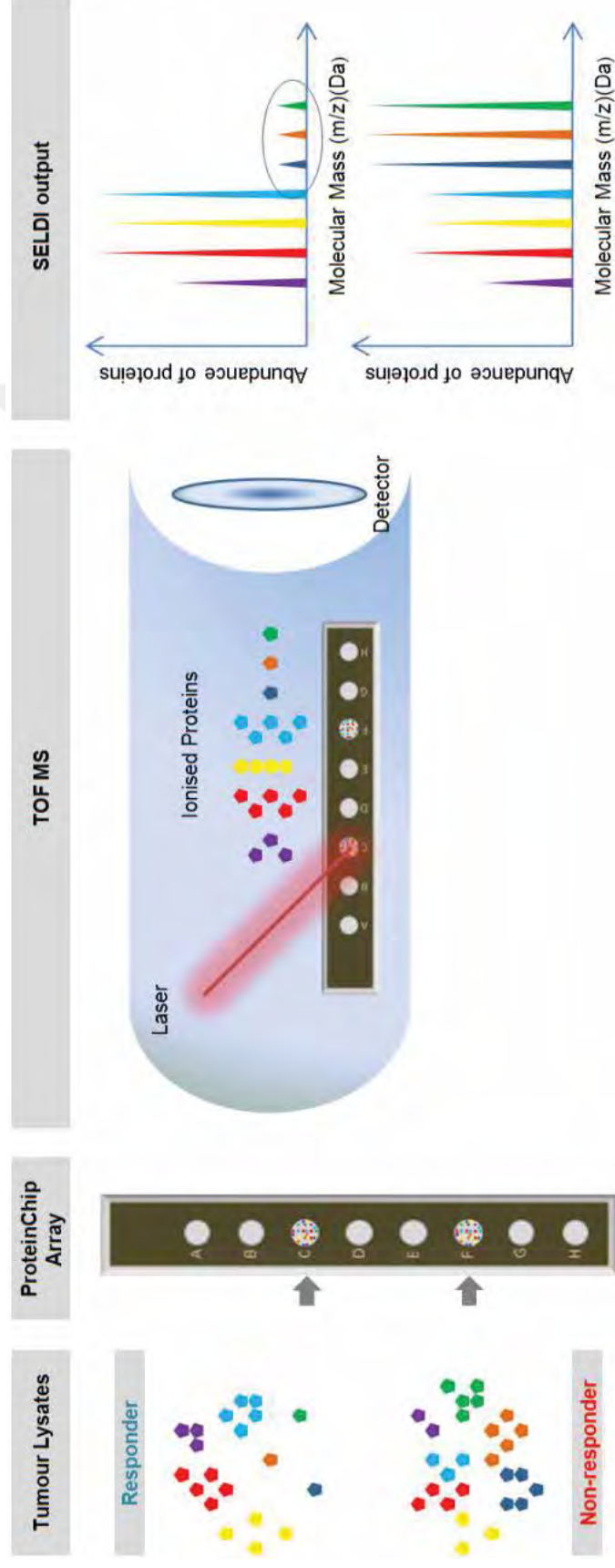


Figure 1.8 Biomarker discovery in patient samples by SELDI-TOF MS using ProteinChip arrays

Crude protein extracts are applied and incubated on the chemically modified array spots then unbound proteins are removed with appropriate buffers. Bound proteins are co-crystallised with energy absorbing molecules and the array spots are irradiated with a laser in order to ionise retained peptides on the array surface. The ions are then accelerated through an electric field in TOF tube where they are differentiated according to their time of flight time. The detector quantifies the proteins and a protein spectrum is generated with calculated m/z ratios on x-axis and peak intensities on y-axis.

Abbreviations: m/z : mass to charge, Da: Dalton, TOF MS: Time of flight mass spectrometry, SELDI: Surface enhanced laser desorption/ionisation

1.4.3 Applications of ProteinChip SELDI-TOF MS Technology in Pathological States

The key applications of the ProteinChip SELDI-TOF MS include the detection of differentially expressed proteins between normal pathological states, biomarker discovery, purification, characterisation of the biomarkers and protein interaction based functional assays (Issaq et al., 2002; Wright, 2002). SELDI-TOF MS as a platform for biomarker discovery has been reported in a variety of diseases such as rheumatoid arthritis (Uchida et al., 2002), cardiovascular diseases (Stanley et al., 2004) and neurological disorders (Lewczuk et al., 2004), ischemic and hemorrhagic stroke (Allard et al., 2004). SELDI-TOF MS has been used extensively in cancer studies. The first study identified a discriminating proteomic pattern in serum samples from patients with ovarian cancer and healthy controls (Petricoin et al., 2002a). Investigations in clinical oncology using SELDI-TOF MS has since revealed potential biomarkers for various types of cancers including prostate bladder, breast, liver, renal, pancreatic, endometrial cancer, follicular and lymphoblastoid lymphoma (Ciordia et al., 2006) The following sections provide more detailed information about applications of SELDI-TOF MS technology in biomarker discovery of various cancer types.

1.4.3.1 Ovarian Cancer

Cancer Antigen 125 (CA125) was introduced by Bast *et al.* more than two decades ago as a monoclonal antibody (OC125) reacting with epithelial ovarian carcinoma cell lines (Bast et al., 1981). CA125 is the only and widely used biomarker recommended for detection of early clinical symptoms of ovarian cancer (Rein et al., 2011). However, elevated levels of this marker can also be detected in endometriosis, adenomyosis and other benign conditions in premenopausal women and produce false-positive results for screening (Bast, 2003). Therefore, CA125 is suggested to be a suitable marker only for

treatment monitoring due to its low sensitivity and specificity for diagnosis (Sturgeon et al., 2008). The potential efficacy of SELDI-TOF MS protein profiling in discovery of ovarian cancer biomarkers with high sensitivity and specificity have been reported by several studies (Kozak et al., 2003b; Yu et al., 2005; Zhang et al., 2004). In a study using a range of serum samples from 361 patients with ovarian cancer, three biomarkers with high sensitivity and specificity were suggested to improve the detection of early stage ovarian cancer. The three biomarkers were identified as apolipoprotein AI (Apo AI), a truncated form of transthyretin (TT) and a cleavage fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) (Zhang et al., 2004). Two of these biomarkers were subsequently confirmed in an independent study and a combination of five serum biomarkers have been shown to be capable of detecting early-stage ovarian cancers with greater efficiency than CA125 alone (Kozak et al., 2005). Because 10-20 percent of ovarian cancers lack expression of CA125, multiple biomarker strategy is required to achieve sequential two-stage screening to detect early-stage ovarian cancer (Clarke et al., 2011; Duffy et al., 2005). A recent study investigated collective discriminative power of CA125 and seven other biomarkers that were previously identified using SELDI-TOF MS and results suggested an improved sensitivity and specificity for combination of Apo AI, TT and Connective Tissue Activating Protein III (CTAPIII) with CA125 to distinguish benign from malignant pelvic masses (Clarke et al., 2011).

1.4.3.2 Breast Cancer

Early detection is known to be crucial to reduce mortality arising from breast cancer (Gast et al., 2009b). Current diagnostic methods such as Cancer Antigen 15.3 (CA15.3) assay which measures Mucin-1 (MUC-1) appear to lack high specificity or/and sensitivity (Baines et al., 1986; Duffy et al., 2010). In addition, distinguishing high-risk patients and orienting personalised therapies remain major challenges in the clinic

(Maurya et al., 2007). SELDI-TOF MS has been applied to a broad range of biological materials including tissue (Traub et al., 2005), serum (Gast et al., 2009c; Li et al., 2002), plasma (Caputo et al., 2005), nipple aspirate fluid (Coombes et al., 2003) and ductal lavage fluid (Li et al., 2005a) obtained from breast cancer patients. A limited number of studies investigated the effects of chemotherapy on protein expression using SELDI-TOF MS technique (Heike et al., 2005; Pusztai et al., 2004). In a study of six breast cancer patients undergoing docetaxel treatment, high molecular weight kininogen and apolipoprotein AII (Apo AII) were identified as two therapy-responsive proteins in blood samples collected before and after docetaxel injection (Heike et al., 2005). Gast and colleagues analysed serum and tissue samples from 26 controls and 75 breast cancer cases on IMAC-Ni and Q10 arrays and detected 27 discriminative peaks between tissue samples of cancer and control groups. Two out of 27 peaks identified as N-terminal albumin fragments are suggested to be products of cancer-specific proteolytic activity in the tumour microenvironment which potentially facilitate diagnosis of breast cancer (Gast et al., 2009a). The same research group also conducted analysis of serum samples from a larger patient cohort (breast cancer: 152 and healthy control: 129) and discovered 10 potential serum biomarkers which might be employed to improve diagnosis (Gast et al., 2009c). These studies indicate the potential of SELDI-TOF MS technology to determine protein profiles of various tumour-associated biological samples for discovery of novel biomarkers.

1.4.3.3 Prostate Cancer

Prostate Specific Antigen (PSA) is a useful marker for early detect and monitoring of prostate cancer progression. However, insufficient specificity of PSA limits its use as a strong early diagnosis biomarker (Qu et al., 2002). Combination of SELDI technology with bioinformatics identified multiple proteins as signature proteomic patterns for the

differentiation of cancer from non-cancer patients (Adam et al., 2002; Banez et al., 2003; Petricoin et al., 2002b; Qu et al., 2002). Petricoin and colleagues conducted a study to determine the potential of proteomic pattern diagnostics to distinguish between patients with prostate cancer and benign prostate conditions (Petricoin et al., 2002b). An algorithm consisted of seven protein masses correctly predicted prostate cancer cases in 95% of samples from patients diagnosed with prostate cancer, including serum samples from individuals whose PSA levels were in the indeterminate range (Petricoin et al., 2002b). However, predictability of this algorithm in classifying patients with marginally elevated PSA levels as having benign conditions remained at 71% which suggests a complementary role for proteomic patterns in conjunction with physical examination, imaging and measurement of serum PSA levels for diagnosis of prostate cancer (Petricoin et al., 2002b). In another study, a nine-protein mass pattern developed from protein profiles of serum samples from 167 prostate cancer, 77 benign prostate hyperplasia patients and 82 healthy individuals has been shown to correctly classify 96% of subjects as prostate cancer patients in a blinded test set (Adam et al., 2002). In addition, an eight-protein biomarker algorithm with unidentified identities indicating a sensitivity of 92% and a specificity of 96.7% was also used as a signature to discriminate between patients with and without prostate cancer (Pan et al., 2006). These studies represent the use of SELDI-TOF MS in developing classification algorithms as an effective approach for early diagnosis of prostate cancer.

1.4.3.4 Colorectal Cancer

Current methods used for colorectal cancer screening comprise colonoscopy and new modalities such as computed tomography colonography, capsule endoscopy, fecal immunochemical test and fecal DNA testing (Cummings and Cooper, 2011). For the detection and removal of polyps, colonoscopy technique is considered as the gold

standard; however, limited sensitivity and specificity have been reported for colonoscopy and alternative techniques in detecting small polyps (< 1cm) (Cummings and Cooper, 2011; de Wijkerslooth et al., 2011). SELDI-TOF MS coupled artificial neural network analysis was used to define a specific algorithm in combination of four protein peaks at m/z 5911, 8930, 8817 and 4476 with 91% sensitivity and 93% specificity to separate colorectal cancer samples from healthy samples (Chen et al., 2004). Engwegen and colleagues discovered a panel of five serum biomarkers in order to discriminate colorectal cancer patients from healthy controls and three of those could be identified as apolipoprotein CI (Apo CI), Apo AI, N-terminal fragment of albumin. Reproducibility of this biomarker panel has been assessed in an independent validation cohort and results indicated 65% sensitivity and 90% specificity in combination of five biomarkers (Engwegen et al., 2006). In a recent study including 42 colorectal cancer patients, serum samples collected prior to treatment and during the course of chemotherapy with oxaliplatin and capecitabine were analysed to discover biomarkers predicting therapy response (Helgason et al., 2010). 13 biomarkers were identified from cancer patients and 6 proteins at m/z 3330, 2756, 6847, 3893, 3978 and 2055 were detected to be potential biomarkers to discriminate between responder and non-responder patient groups (Helgason et al., 2010). Application of SELDI-TOF MS for detection of cancer and biomarker discovery contributes to reliably distinguish cancer patients from healthy population or to monitor response of patients to therapies.

1.4.3.5 Pancreatic Cancer

Tumour associated carbohydrate antigen, CA19-9, is the most widely used serum marker to differentiate pancreatic cancer from chronic or relapsed pancreatitis (Rückert et al., 2010). However, CA19-9 is insufficient for screening asymptomatic individuals with pancreatic cancer (Rückert et al., 2010). A number of studies have reported the use

of SELDI-TOF MS technology for biomarker discovery in early detection of pancreatic cancer (Ehmann et al., 2007; Honda et al., 2005; Koopmann et al., 2004; Liu et al., 2009; Takano et al., 2008). Koopman and colleagues demonstrated that protein profiling of serum samples from pancreatic adenocarcinoma (n=60), non-malignant pancreatic diseases (n=60) and healthy controls (n=60) revealed two discriminative markers with 78% sensitivity and 97% specificity which were significantly ($p < 0.05$) superior than CA19-9 to differentiate pancreatic cancers from healthy controls (Koopmann et al., 2004). The SELDI-TOF MS study of Honda *et al.* developed an algorithm of four differentially expressed plasma proteins (m/z 87566, 17272, 28080 and 14779) to correctly discriminate pancreatic cancer patients from healthy controls with 97.2 % sensitivity and 94.4 % specificity in a training cohort. Discriminative ability of this algorithm was verified with 90.9% sensitivity and 91.1% specificity in a validation sample set. Combined predictability of these biomarkers with CA19-9 was also examined in an independent validation cohort. Even though classification of healthy samples resulted in six false-positive cases corresponding to 15.4% of healthy controls, complementary use of CA19-9 (86.2%, alone) and SELDI biomarkers (89.7%, alone) correctly classified 100% of cancer patients into pancreatic cancer group (Honda et al., 2005).

1.4.3.6 Applications of ProteinChip SELDI-TOF MS Technology in Glioma and Other Brain Tumours

During the last decade, a wide range of technologies has been employed in molecular and genetic profiling studies for biomarker discovery in brain tumours. However, a limited number of biomarkers have been identified and none of these biomarker candidates can be translated into clinics (Kalinina et al., 2011b). Therefore, new approaches for biomarker discovery are needed.

In a SELDI-TOF MS study of 105 serum samples from 28 patients with astrocytoma [Grade I, II, III-Anaplastic Astrocytoma (AA), Grade IV (Glioma)] 37 patients with benign tumour and 40 healthy individuals, artificial neural network algorithm was used to select biomarkers based on protein profile analysis. While two thirds of samples were used as a training set, one third of entire cohort was utilised to be a test set to assess power of selected potential biomarkers. Combination of 15 peaks discriminated patients with astrocytoma from healthy cases by 95.7% (true positives divided by the total number of individuals with the disease; sensitivity: 88.9% and true negatives divided by the number of subjects without the disease; specificity: 100%) accuracy. 86.4% discriminative ability was obtained when 20 peaks were used in combination to differentiate between astrocytoma and benign brain tumour samples. When low grade astrocytoma (Grade I-II) was compared with high grade astrocytoma (AA-Glioma), an accuracy of 85.7% was achieved by a combination of three peaks (Liu et al., 2005). In another study including 140 serum samples, a combination of seven serum biomarkers with unknown identity has been suggested to be a significant classifier with 84.6% sensitivity and 86.4% specificity to correctly separate astrocytoma cases from healthy controls. Among these biomarkers four (at m/z 2018, 6633, 7567, 15115) were up-regulated and three were down-regulated (at m/z 4286, 8140, 8926) in astrocytoma patients. In addition, significantly higher expression of two of these proteins, m/z 7567 and 15115, were detected in low grade astrocytoma compared with high grade astrocytoma (Zhang et al., 2007a). Using SELDI-TOF MS technique, Petrik *et al.* detected a 2740 Da protein peak in mass spectra analysis from 200 serum samples (training cohort) of 58 healthy individuals, 36 patients with grade II astrocytoma, 15 with AA and 91 with glioma. The protein peak was found to be less abundant with the increasing astrocytoma grade and identified as β -chain of α_2 -Heremans-Schmid

glycoprotein (AHSG). Moreover, serum levels of AHSG were validated in the training cohort as well as in an independent set of serum samples from 72 glioma patients using a turbidimetric method and results suggested AHSG as an independent predictor of patient survival. While low serum levels of AHSG were correlated with short median survival time (< 3 months), normal levels in serum were found to be associated with prolonged survival (> 2 years) (Petrik et al., 2008).

In addition to the SELDI-TOF MS applications for biomarker discovery in serum samples, Khwaja and colleagues investigated cerebrospinal fluid (CSF) collected from 32 patients, comprising 10 with neoplastic CNS conditions, 12 with inflammatory or reactive conditions and 10 control samples without any significant CNS disease. 10 neoplastic samples included Ependymoma ($n = 1$), astrocytoma grade II ($n = 1$), glioma grade IV ($n = 5$), melanoma ($n = 2$), metastatic carcinoma ($n = 1$). Analysis of protein profiles revealed 30-32 kDa protein overexpressed in neoplastic samples including high grade gliomas. This candidate biomarker was subsequently identified as carbonic anhydrase which functions in proliferation, survival and invasion of cells by regulating cellular and tissue acid-base homeostasis (Khwaja et al., 2006).

Several SELDI-TOF MS studies have investigated protein profiles of tissue samples obtained from surgical resection (Hobbs et al., 2003) and animal models (Bouamrani et al., 2006a; Wibom et al., 2006). In a study comparing differential protein expression of contrast-enhanced (CE) and non-enhanced (NE) regions of the tumour specimens from four glioma patients, correlation between MR contrast-enhancement signatures and protein expression patterns were evaluated. Protein profiles of CE regions indicated heterogeneity within the same tumour. In contrast, similar protein expression patterns were reported for NE regions in the same tumour and also between individuals.

Increased expression of small molecular weight proteins was detected in CE regions when compared to the NE regions. These findings suggested that protein fingerprints from CE and NE regions were correlated with contrast enhancement imaging signatures (Hobbs et al., 2003).

Wibom et al. conducted a SELDI-TOF MS study to explore differentially expressed proteins in single fraction 12 Gy radiation-treated ($n = 12$) and untreated ($n = 12$) BDIX-rats implanted with rat BT4C glioma cells. Two multivariate projection methods, principal component analysis (PCA) and partial least squares (PLS) were used to analyse mass spectral data. Distinct protein patterns were reported between the analysis of tumour and normal tissue. 77 protein peaks differentially expressed between irradiated and non-irradiated tumour samples have been suggested to generate potential biomarker clusters in defining radiation-induced changes and developing new approaches for glioma treatment (Wibom et al., 2006).

To conclude, SELDI-TOF MS is a sensitive and effective proteomic technology particularly for the analysis of small molecular weight proteins (Liu, 2011). SELDI-TOF MS technology has been utilised in protein profiling analysis of a variety of specimens derived from patients with brain tumours for the discovery of biomarkers which facilitate diagnosis, predict therapeutic response and determine grading of tumours. Since there is a growing need for the development of diagnostic, predictive or prognostic biomarkers, in this study we investigated differentially expressed proteins in tissue samples of glioma patients using SELDI-TOF MS technology for global protein profiling.

1.4.3.7 Other Cancers

SELDI-TOF MS technology was also applied for biomarker discovery studies in a variety of other cancers including renal cell carcinoma (Engwegen et al., 2009; Xu et al., 2009), melanoma (Wilson et al., 2004), cervical cancer (Panicker et al., 2009a), lung cancer (Han et al., 2008), head and neck cancer (Wadsworth et al., 2004), liver cancer, follicular lymphoma (Lin et al., 2004) and hepatocellular carcinoma (Poon et al., 2003). Tumour samples, serum and/or body fluids obtained from cancer patients were analysed in parallel to a control group in order to define protein expression alterations and discover potential biomarkers for diagnosis or monitoring of cancer progression using SELDI-TOF MS technology. SELDI-TOF MS can be utilised to achieve new modalities in diagnosis, predicting therapy outcome and monitoring therapy response for cancers (Liu, 2011).

1.5 AIMS OF THIS THESIS

The broad aim of this thesis is to investigate the contributions of single biomarker candidates, TopoII α , NG2 and MGMT to chemotherapy resistance when treated with etoposide, cilengitide and temozolomide respectively, using human glioma cell lines. These potential targets, TopoII α , NG2 and MGMT were selected due to their critical roles in mechanism of action of therapeutics employed in standard therapy (TMZ) and salvage therapy (etoposide and cilengitide) for glioma treatment. Etoposide, cilengitide and TMZ become key therapeutic agents which are predominantly and currently used in the treatment of glioblastoma. Ultimately, a global proteomic approach using a mass spectrometry based method, SELDI-TOF MS will be utilised in order to discover novel biomarkers in predicting patient response to standard chemotherapy regimen.

To achieve these goals, the specific aims were:

1. To examine the cytotoxic effect of etoposide on glioma cells lines and assess the potential association between the levels of TopoII α expression and response of the cell lines to etoposide treatment.
2. To identify IC₅₀ doses of cilengitide in glioma cell lines to assess the relationship between cilengitide response and expression of NG2 protein which involves in integrin-related chemotherapy resistance.
3. To establish response characteristics of glioma cell lines by determining IC₅₀ doses of TMZ treatment and to assess whether these results are associated with MGMT methylation status or/and expression level of MGMT protein in the cell lines.
4. To analyse crude protein extracts of patient tumour specimens using a mass spectrometric method, SELDI-TOF MS, to identify biomarkers which might distinguish responder patients from non-responders to standard therapy and further might discriminate short term surviving patients from patients who have displayed long term survival.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter describes general methods and materials used within this thesis. Specific methods are detailed in the relevant chapters and the suppliers of materials are listed when it is first mentioned in the text.

2.2 CELL CULTURE METHODS

2.2.1. Maintenance of Cell Lines

Glioma cell lines (T98G, A172, M059K, M059J and U-87 MG) were obtained from the American Tissue Culture Collection (ATCC). An osteosarcoma cell line, MG-63, was kindly provided from Hormones and Cancer Laboratory, Kolling Institute of Cancer Research, Sydney and used as a positive control for NG2 proteoglycan expression. All cell lines were maintained according to the supplier's instructions. Recommended cell culture media were supplemented with essential medium components and 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Castle Hill, Australia). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C (standard incubation conditions). Cell culture plates (6-well, 96-well) and flasks (T25, T75, T175) (Nunc; Thermo Scientific,

Waltham, MA, USA) were used to grow cells. For cell line maintenance, cells with 80% confluency were washed once with pre-warmed sterile PBS and detached with 0.25% trypsin, 0.53 mM Ethylenediaminetetraacetic acid (EDTA) (Amresco, Solon, OH, USA) solution. Harvested cells were centrifuged at 500 x g for 1.5 minute and resuspended in a fresh media for subcultivation.

Glioma neural stem cells (GNS) were kindly provided from the Queensland Institute of Medical Research (QIMR). For the subculturing of cell lines, the cells were washed with DPBS (5 ml) and incubated with accutase (2 ml; Merck Millipore, Billerica, MA, USA) for 5min at 37°C. Following incubation, trypsin inhibitor from Glycine max (1 ml; Sigma-Aldrich, St Louis, MO, USA) was added to inactivate the digesting enzymes. The cell suspension was centrifuged for 5min at 233 x g at 20°C and the supernatant was discarded. The pellet was resuspended in RHB-A stem cell culture medium mixed with β -FGF (20 ng/ml of medium) and EGF (20 ng/ml of medium). The cells were cultured in Nunc flasks T75 and incubated in a Forma Direct Heat Incubator (37°C, 20% O₂ and 5% CO₂, Model 311, Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2 Cryopreservation Technique

Cells were collected as described in Section 2.2.1 and resuspended in freezing media which contained 20% FBS and 20% Dimethylsulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA). Following resuspension, media was aliquoted into 2ml cryovials and stored in -80 °C for 24 hours before transferring to storage in the liquid nitrogen tanks. For recovery of frozen cells, vials were rapidly warmed to 37 °C and added to the prewarmed media containing 10% FBS (Sigma-Aldrich, St Louis, MO, USA). Cells were centrifuged and the cell pellet was resuspended in fresh media then incubated using standard conditions.

2.2.3 Cell Proliferation Assay

To assess cell proliferation, the CellTiter 96® Aqueous Assay (MTS; Promega Corporation, Madison, WI, USA) as described by the manufacturer was used. The MTS assay uses a combination of tetrazolium salt [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and an electron coupling reagent phenazine methosulfate (PMS) (Sigma-Aldrich, St Louis, MO, USA) as an indicator of mitochondrial function in viable cells. All experiments were performed in flat bottom 96-well plates (Nunc; Thermo Scientific, Waltham, MA, USA). MTS reagent is reduced to a coloured and water-soluble formazan product by metabolically active cells. The assay was conducted by adding pre-warmed reaction mixture (100 µl) (MTS-PMS-Media) into each well using a multi-channel pipette. Combination of reagents is shown in Table 2.1. Plates were incubated for 30 to 60 min at 37 °C in a humidified, 5% CO₂. The absorbance of the formazan is measured at 490nm spectrophotometrically using Tecan™ Sunrise® Special Plate Reader (Tecan Group Ltd., Männedorf, Switzerland) and Magellan3™ software. Control wells containing culture media without cells were included in each plate for the normalisation of measurements with subtraction of background absorbance. Experiments were performed three times in triplicates.

Table 2.1 MTS assay mixture for one 96 well plate

Assay Components	Volume (ml)
Media	7.92
FBS	0.88
MTS	2.20
PMS	0.11

Abbreviations: FBS: fetal bovine serum, MTS: 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, PMS: phenazine methosulfate

2.2.4 Cell Density Optimisation and Chemosensitivity Assays

Optimal cell density for each cell line was determined for the drug cytotoxicity assays. Dilutions of cell suspensions from 100 to 5×10^4 cells were seeded in 96-well plates (Thermo Scientific, Waltham, MA, USA) in triplicate. The MTS (see Section 2.2.3) assay was conducted at 24, 48 and 72 hr time points after seeding. When the optimal cell densities were established for cell lines, chemosensitivity of cells to the several chemotherapy drugs (etoposide, TMZ and cilengitide) were assessed and the Half Maximal Inhibitory Concentration (IC_{50}) value calculated. The number of cells/well were dispensed into the plates and incubated for 24 hr in standard incubation conditions. Plates included both 'medium only' and 'medium with cells' control groups as well as multiple dilutions of the chemotherapy drugs which were added after a 24 hr incubation period (see Figure 2.1). MTS assay was performed at 24, 48 and 72 hr post-treatment to determine the cytotoxic effects of the chemotherapy drugs.

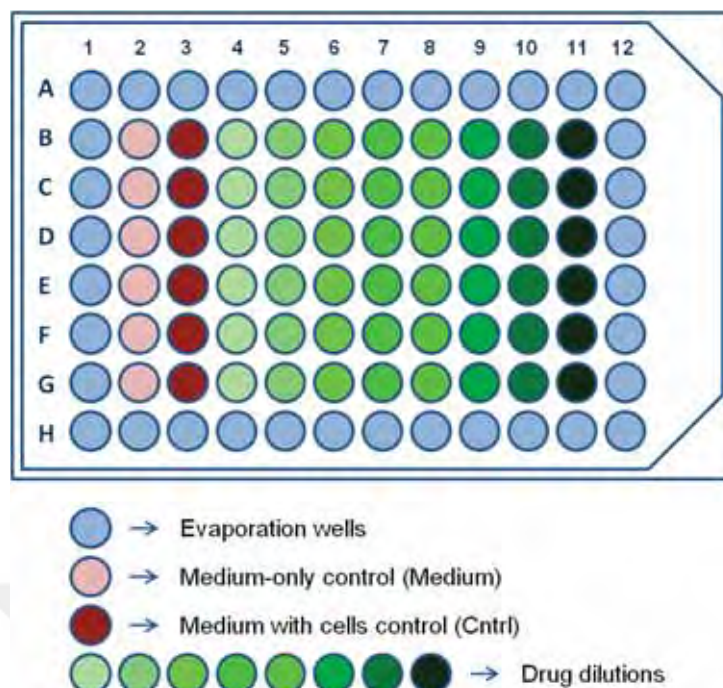


Figure 2.1 A sample design of cytotoxicity assays as illustrated

36 peripheral (outside) wells were filled with PBS to prevent cells from the effect of evaporation on cell viability. Each experiment included 'medium-only' and 'medium with cells' control wells. 6-wells containing 'medium-only' were used to blank absorbance measured at 492nm for MTS assay. 'Medium with cells' control was seeded to monitor drug-free proliferation rates of cells.

2.2.5 Determination of IC_{50}

The average absorbance of medium-only wells was subtracted from the absorbance of media with cells and treatment wells. Values were plotted on the graph with measured absorbance versus drug concentration. The drug dilution value on y-axis corresponding with 50% of the absorbance of cells only control on x-axis indicated the half of the cells which were viable in the applied drug dose (see Figure 2.2).

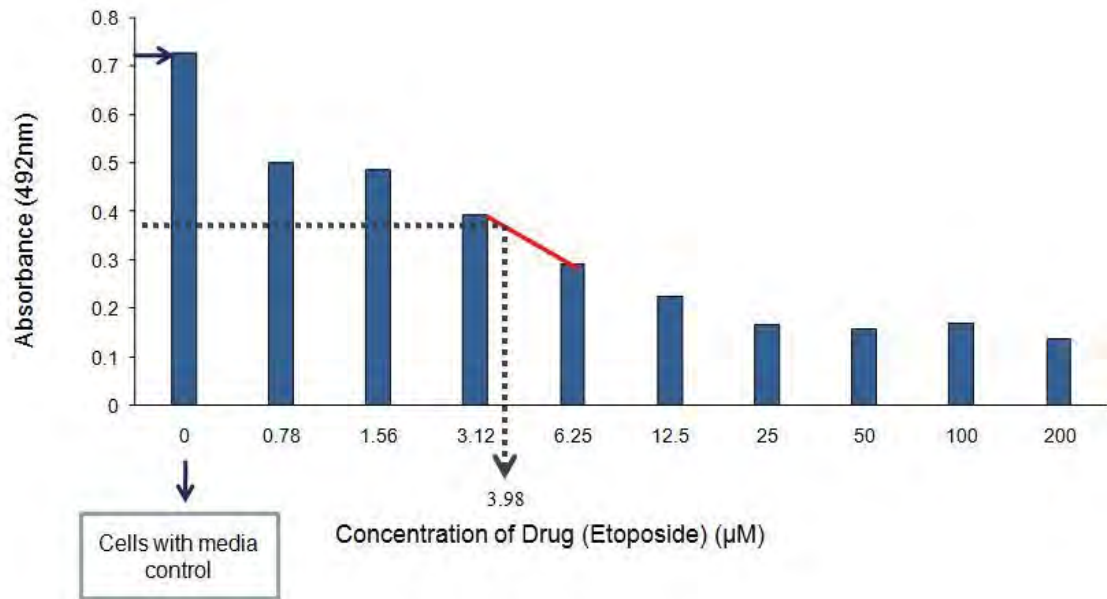


Figure 2.2 An example of dose-response graph demonstrating IC₅₀ value

Formazan production per well was measured at 492 nm following exposure of cells to a wide range of drug concentrations. 50% of the control cell absorbance was used to determine IC₅₀ dose for each cell line.

2.2.6 Trypan Blue Cell Counting

Trypan blue dye was used to stain and distinguish non-viable from viable cells. While non-viable cells absorb trypan blue through their permeable membrane, intact membrane of viable cells exclude the dye. Cells in 80% confluent flasks were trypsinised and centrifuged to obtain a pellet as described in Section 2.2.1. Cells were then resuspended in serum-free complete medium (1 ml). Cell suspension (20 µl) was diluted 1:20 in serum-free complete medium to final volume of 400 µl. Diluted cell suspension (10 µl) and 10 µl of 0.4% trypan blue were mixed in a centrifuge tube and cells were counted within 3 minutes of mixing, as longer incubation leads to cell death. A small amount of stained cell suspension was transferred into both chambers of

haemocytometer covered with a coverslip. After the haemocytometer was filled by capillary action, four corner squares of each chamber were counted and an average viable cell number per square was multiplied by dilution factors and 10^4 (the volume correction factor for the haemocytometer to calculate cells per millilitre). Following formulation was used to calculate number of cells/ml for above protocol.

$$\text{Cell/ml} = \text{Average cell count per square} \times 20 \text{ (dilution factor)} \times 2 \text{ (dilution factor)} \times 10^4$$

2.3 DNA METHODS

Topic-specific methodologies are detailed in relevant chapters. Therefore, DNA methods were described in Chapter 5.

2.4 RNA METHODS

All equipment and bench areas dedicated to RNA work were cleaned with RNase-Zap prior to use. RNase-free reagents, solutions and materials were used in RNA work.

2.4.1 RNA Extraction from Cultured Cells

All cell lines were routinely grown as monolayer cultures. Cells were washed twice with sterile, ice-cold phosphate buffered saline (PBS) solution. Cells were directly lysed by adding TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) into the culture, 750 μ l for each well of 6-well plate and 3 ml for T75 flask. A sterile cell scraper was used to pool the cells to one corner of the T75 flask and cell lysate was transferred into a 2 ml RNase-free centrifuge tube (Scientific Specialties Inc., Lodi, CA, USA). 200 μ l of chloroform (Crown Scientific, Minto NSW, Australia) was added and the tube was shaken vigorously for 15 seconds. The tube was then incubated at room temperature for 10 minutes and centrifuged at 12000 x g for 15 minutes at 4 $^{\circ}$ C using a refrigerated centrifuge (Eppendorf, Hamburg, Germany). After the centrifugation clear upper

aqueous phase including RNA was added to the tube containing isopropanol (500 µl) (Bacto Laboratories, Mt Pritchard, NSW, Australia) and mixed by gentle inversion. The tube was incubated again at room temperature for 10 minutes and centrifuged at 12000 x g for 8 minutes at 4 °C to obtain the pellet. The supernatant was discarded and the pellet washed with 75% ethanol then the tube was mixed by inverting the tube 5-6 times followed by centrifugation at 7500 x g for 5 minutes at 4 °C. The supernatant was removed as much as possible and centrifuged again at 7500 x g for 1 minute at 4 °C. Then the pellet was dried by evaporation of ethanol. The dry RNA pellet was resuspended in 20 µl of RNase free water. RNA was quantified by NanoDrop spectrophotometer (NanoDrop® ND-1000, Wilmington, DE, USA) and the sample was stored at -80 °C until required.

2.4.2 Lithium Chloride (LiCl) Precipitation of RNA

Total RNA extracted as described in Section 2.4.1 was diluted to a concentration of 1µg/µl. RNA (100 µl) and LiCl (50 µl) (Applied Biosystems/Ambion, Austin, TX, USA) were mixed (final concentration of LiCl 2.5 mol/L), vortexed thoroughly and incubated for 2 hours at -20 °C. After the incubation, the tube was centrifuged at 15000 x g for 10 minutes at 4 °C and the supernatant was discarded. 75% ethanol (1ml) was added to the pellet and centrifuged at 15000 x g for 10 minutes at 4 °C. After the removal of the supernatant, 75% ethanol (1ml) was added, mixed by vortexing and centrifuged at 15000 x g for 10 minutes at 4 °C. Ethanol was discarded and the pellet was allowed to dry for 1-3 minutes to evaporate any remaining ethanol. The air-dried pellet was dissolved in RNase free water and the concentration of RNA was measured by NanoDrop spectrophotometer (NanoDrop® ND-1000, Wilmington, DE, USA). RNA sample was stored at -80 °C.

2.4.3 cDNA Synthesis

The reverse transcription of mRNA into cDNA was performed using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) in two steps. RNA from cell lines was prepared as described in Section 2.4.1 and 2.4.2. Final concentration of RNA samples was adjusted to 1000 ng/μl. In addition to RNAs extracted from cell lines, commercial Human Normal Brain Reference RNA samples; Human Parietal Cortex Superior Brain Total RNA (NBPCS) (1 mg/ml) (10μg, Ambion Cat#6790, Applied Biosystems/Ambion, Austin, TX, USA), Human Brain Reference RNA (NBTot) (1 mg/ml), (200 μg, Ambion Cat# 6051, Applied Biosystems/Ambion, Austin, TX, USA), Human Orbital Frontal Cortex Brain Total RNA (NBORF) (1 mg/ml) (50 μg, Ambion Cat#AM6786, Applied Biosystems/Ambion, Austin, TX, USA), Human Parietal Cortex Posterior Brain Total RNA (NBPCP) (1mg/ml) (50 μg, Ambion Cat#AM6788, Applied Biosystems/Ambion, Austin, TX, USA) were prepared for cDNA conversion. For the first step, random hexamers (1 μl), dNTP mix (1 μl) and DEPC-treated water (9 μl) were mixed with RNA sample (1 μl). The tube was briefly mixed then placed into the MJ DNA Engine, DYAD, Peltier Thermal Cycler (MJ Research, Waltham, MA) to incubate the mixture at 65 °C for 5 minutes. After incubation, second step of the conversion was performed by mixing reagents detailed in Table 2.2 and 12 μl of reaction mixture from the first step. The tube was mixed gently and spun briefly then incubated in the Thermal Cycler with the settings listed in Table 2.3, step II.

Table 2.2 SuperScript® III First-Strand Synthesis System components

Reagents	Amount (µl/reaction)
Random hexamers (50 ng/µl)	1
10X RT buffer	2
25 mM MgCl ₂	4
0.1 M DTT	2
10 mM dNTP mix	1
SuperScript III RT (200 U/µl)	1
RNaseOUT (40 U/µl)	1
DEPC-treated water	9

Abbreviations: DEPC: Diethylpyrocarbonate, dNTPs: deoxynucleotide triphosphates, DTT: dithiothreitol, MgCl₂: magnesium chloride, U: units

Table 2.3 PCR conditions of cDNA synthesis

Steps	Conditions
I	65 °C for 5 minutes
	25 °C for 10 minutes
II	50 °C for 50 minutes
	85 °C for 5 minutes

2.4.4 Control for Genomic DNA Contamination of RNA Samples

cDNAs were synthesised as described in Section 2.4.3 and assessed for genomic DNA contamination by PCR amplification of the housekeeping gene β -Glucuronidase (*GUS- β*) transcript using primers *GUS- β 3'* and *GUS- β 5'*. cDNA (1 μ L) was mixed with master mix (46 μ l) (detailed in Table 2.4) and AmpliTaq Gold® DNA Polymerase (3000 U) (0.5 μ l) (Applied Biosystems, Foster City, CA, USA) and the tubes containing sample mixture were placed into the MJ DNA Engine, DYAD, Peltier Thermal Cycler (MJ Research, Waltham, MA) then, amplified using the program detailed in Table 2.7 above.

Table 2.4 PCR components

Reagents	Amount (μ l/reaction)
Nuclease Free Water	36
10X PCR buffer II	5
dNTPs (2.5 mM, diluted from 100mM)	1
<i>GUS-β3'</i>	1
<i>GUS-β5'</i>	1
MgCl ₂ (25 mM)	2
AmpliTaq Gold® DNA Polymerase (3000U)	0.5
cDNA Sample	1

Abbreviations: dNTPs: deoxynucleotide triphosphates, *GUS- β* : β -Glucuronidase, MgCl₂: magnesium chloride, U: units

To assess genomic DNA contamination, polyacrylamide gel electrophoresis was performed as described in Section 2.3.8. Presence of contaminating DNA in RNA samples was observed (See Figure 2.3, A). After contamination was confirmed, LiCl precipitated RNA samples were subjected to cDNA conversion, *GUS- β* DNA synthesis. The presence of a band at ~400 bp indicated contamination of the samples with genomic

DNA. Following LiCl precipitation of samples, only one DNA band at ~200 bp was detected reflecting clean RNA samples which were suitable to perform qRT-PCR. (Figure 2.3, B).

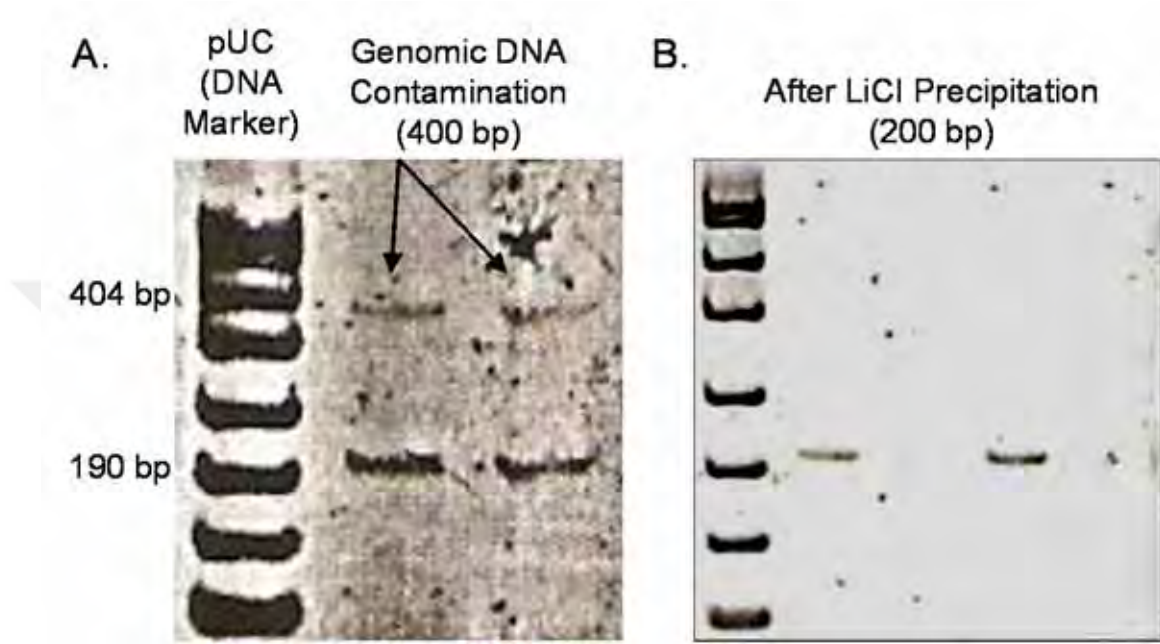


Figure 2.3 *GUSβ* DNA amplification to control for genomic DNA contamination of the cDNA samples converted from RNAs.

The presence of a band at ~400 bp confirmed contamination of the cDNA with genomic DNA. **(A)** Amplified *GUSβ* DNA which generates a ~400 bp product with a genomic DNA template and a ~200 bp band with cDNA template. **(B)** Only expected size of DNA amplified after LiCl precipitation of RNA samples.

2.4.5 Quantitative Real-Time PCR (qRT-PCR)

RNA expressions of specific target genes were analysed using qRT-PCR. mRNA transcripts were first reverse transcribed into cDNA as detailed in Section 2.4.3. A master mix containing Taqman gene expression assay-Gene-specific TaqMan® probe and primer sets-(Applied Biosystems, Foster City, CA, USA) and Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems, Foster City, CA, USA) (see Table 2.5) was prepared as detailed in Table 2.6. cDNA was mixed with the master mix in 100μl strip

tubes (Corbett Research, San Francisco, CA, USA) using Eppendorf epMotion 5070 automated pipette robot. Samples were prepared in triplicate and each assay was repeated three times. All reactions were performed on a RotorGene 3000 (Corbett Research, San Francisco, CA, USA) using settings listed in Table 2.7. The fold change in the expression level of the gene was calculated using $\Delta\Delta C_t$ method on Relative Expression Software Tool (REST) Software (Corbett Research, San Francisco, CA, USA).

Table 2.5 Details of qRT-PCR assays

Target Gene	TaqMan PCR MasterMix	Taqman Gene Expression Assay ID
<i>Topolla</i>	TaqMan Universal Master Mix	Hs00172214_m1 (TOP2A)
<i>NG2</i>	TaqMan Gene Expression Master Mix	Hs00426981_m1 (CSPG4)
<i>18S</i>	TaqMan Universal/Gene Expression Master Mix	VIC/MGB Probe

Table 2.6 Components for the qRT-PCR reaction mix

Components	Amount(μ l)/Reaction	Final concentration
RNase-Free Water	6	-
2X TaqMan Universal PCR MasterMix	10	1X
20X Target Primers and Probe	1	1X
20X Control Primers and Probe	1	1X
cDNA Sample	2	-
Final Volume	20	1X

Table 2.7 Thermal cycling programs

Steps	Temperature	Duration of the Step
1	95 $^{\circ}$ C	10 minutes
2	95 $^{\circ}$ C	10 seconds
3	60 $^{\circ}$ C	45 seconds
4	Step 2-3 repeated 40 cycles	

2.5 PROTEIN METHODS

2.5.1 Protein Extraction from Glioma Cell Lines

Cell lysis buffer (Dithiothreitol [DTT] and Bromo-phenol Blue) was prepared to collect the cell lysate by combining reagents listed in Table 2.8. One cOmplete Mini Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN, USA) was dissolved in MilliQ water and added to the 10 ml of lysis buffer separately just before cell harvesting. Cells grown to 80% confluency were washed twice with ice-cold PBS. 1 ml of lysis buffer for T75 flasks and 200 μ l for each well of 6-well plate were directly added to the cell monolayer. Cells were harvested using a cell scraper and transferred into a 15 ml sterile centrifuge tube. Cell lysate was then sonicated for 15-30 seconds until the cell clumps were dissolved and the lysis was completed. After sonication, the sample was transferred into a sterile 1.5 ml centrifuge tube and denatured at 95 $^{\circ}$ C for 10 minutes in a heat block then centrifuged at 10000 x g for 5 minutes. The supernatant was transferred into a clean centrifuge tube and dispensed into 50 μ l aliquots, then stored at -80 $^{\circ}$ C.

Table 2.8 Components of protein extraction buffer

Reagents	Volume-Weight	Final Concentration
1 M Tris-HCl pH 6.8	6.25 ml	125 mM
10% SDS	10 ml	2%
100% Glycerol	5 ml	10%
MilliQ water	To 50 ml	
cOmplete Mini Protease Inhibitor Cocktail		
DTT*		
Bromo-phenol blue*		

* DTT is unstable in protein lysis buffer. * Present concentrations of bromo-phenol blue in lysis buffer interfered with the result of protein assay. These reagents were added immediately before immunoblotting after the quantification of sample.

2.5.2 Determining Protein Concentration

A series of dilutions of Bovine Serum Albumin (BSA) (Genesearch Pty. Ltd., Aundel, QL, Australia) was prepared in lysis buffer with final concentrations detailed in Table 2.9. DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure concentration of cell extracts. To prepare Reagent A+S, 20 µl of Reagent S was added to each ml of reagent A in a centrifuge tube. BSA standards (5 µl) and protein samples (5 µl) were transferred into a clean microtiter plate in duplicate. Then, Reagent A+S (25 µl) and Reagent B (200 µl) were added into each well. The microplate was vigorously shaken for 5 seconds on Tecan™ Sunrise® Special Plate Reader (Tecan Group Ltd., Männedorf, Switzerland) using Magellan3™ software then incubated for 15 minutes. Following incubation, absorbances were measured at 650 nm.

Table 2.9 Dilutions of BSA protein standard

Protein Standards	BSA Concentration (mg/ml)
1	1.500
2	1.000
3	0.660
4	0.440
5	0.296
6	0.132

Abbreviations: BSA: Bovine Serum Albumin

2.5.3 Immunoblotting

30 µg of protein lysate in a total volume of 20 µl (detailed in Table 2.10) was loaded per lane of the NuPAGE gels (Invitrogen, Carlsbad, CA, USA) placed in XCell SureLock™ Mini-Cell Electrophoresis System which was pre-filled with running buffer. Electrophoresis for each gel type was performed at different conditions listed in Table 2.11). Three different protein standards were used to estimate molecular weights of the

proteins accurately; Kaleidoscope Precision Plus (Bio-Rad, Hercules, CA, USA) for TopoII α , HiMark™ Pre-stained Protein Stain (Invitrogen, Carlsbad, CA, USA) for NG2, SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, Carlsbad, CA, USA) (see Appendix C) for DEFA3, MIF and S100A8.

Table 2.10 Components of protein mix for gel loading

Reagents	Volume (μ l)	Final Concentration
Protein in lysis buffer	x	
Lysis buffer	18-x	
1 M DTT	1	50 mM
0.2% Bromo-phenol solution	1	0.01%
Final volume	20	

Following electrophoresis, proteins separated on gels were transferred onto Amersham Hybond™ ECL™ Nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using semi-dry and wet transfer techniques.

2.5.3.1 Semi-dry Transfer of Proteins

The gel containing separated proteins/nitrocellulose membrane/filter sandwich was prepared by pre-wetting each of these components with Schafer-Nielson Semi-Dry Transfer Buffer (20% Methanol) (see Appendix B) then placed directly on the anode plate (platinum-coated titanium) of TransBlot SD SemiDry transfer unit (BioRad, Hercules, CA, USA) in a horizontal configuration. The blotting roller was used to remove any air bubbles trapped between the gel and the membrane. The top cathode plate was aligned over the bottom anode electrode plate then the proteins were transferred at 15 V for 60 minutes.

2.5.3.2 Wet Transfer of Proteins

The filter papers, the gel and the Amersham™ Hybond™ ECL Nitrocellulose Membrane (GE Healthcare, Buckinghamshire, UK) were soaked with NuPAGE transfer buffer (20% methanol) and sandwiched between wetted sponge pads. The transfer sandwich was then transferred to the gel holder cassette and placed in the mini tank (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, Hercules, CA, USA). The tank was filled up with a transfer buffer and a cooling unit was also inserted in the tank. The transfer was performed under the conditions detailed in Table 2.11.

After the protein transfer was completed, the membrane was separated from the other components of the sandwich and quickly washed with TBST. Following transfer, the membrane was placed in a sufficient amount of Ponceau S Staining Solution (Sigma-Aldrich, St Louis, MO, USA) and shaken until the reddish pink stained bands were visible. This reversible staining method allows further immunological detection. Afterwards the membrane was de-stained in milliQ water by continued washing until the background was clean. Blocking buffer (see Appendix B) was then applied for 3 hr at room temperature. Details of the incubations periods and the dilutions of the primary and HRP conjugated secondary antibodies were listed in Table 2.12. A series of washing steps (three times each for 5 min and three times each for 15 min) in TBST was performed between antibody incubations to remove unbound antibody. All incubations and washing steps were performed on a horizontal platform shaker with continuous, gentle rotation. A housekeeping gene, monoclonal anti- α -tubulin clone DM1A (Sigma-Aldrich, St Louis, MO, USA) was used as a loading control. Blots were then developed using Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions and imaged on a

LAS-4000 (Fuji Photo Film Co.Ltd., Tokyo, Japan). Densitometric analyses of the blots were performed using Multi Gauge software V3.0 (Fuji Photo Film Co.Ltd., Tokyo, Japan).

Table 2.11 Details of electrophoresis and transfer steps for different target proteins

Proteins		
TopoIIα (180 kDa)	Gel Type	NuPAGE Novex 4-12 % Bis-Tris Gel
	Running Buffer	1X The NuPAGE® MOPS SDS Running Buffer
	Transfer Buffer	Semi-dry transfer buffer (see Appendix B)
	Conditions	Running at 150 V for 1hr, Semi-dry Transfer at 15 V for 1hr
NG2 (260 kDa)	Gel Type	NuPAGE Novex 3-8 %Tris-Acetate Gel
	Running Buffer	1X NuPAGE Novex Tris-Acetate SDS running buffer
	Transfer Buffer	NuPAGE Transfer Buffer
	Conditions	Running at 150 V for 1 hr, wet transfer at 90 V for 3 hr at 4 °C
MGMT (23 kDa)	Gel Type	NuPAGE Novex 12 % Bis-Tris Gel
	Running Buffer	1X The NuPAGE® MOPS SDS Running Buffer
	Transfer Buffer	NuPAGE Transfer Buffer
	Conditions	Running at 200 V for 30 min, wet transfer at 90 V for 3 hr at 4 °C
S100A8 (10.8 kDa)	Gel Type	NuPAGE Novex 12 % Bis-Tris Gel
	Running Buffer	1X The NuPAGE® MES SDS Running Buffer
	Transfer Buffer	NuPAGE Transfer Buffer
	Conditions	Running at 180 V for 40 min, wet transfer at 90 V for 90 min
DEFA3 (10.2 kDa)	Gel Type	NuPAGE Novex 12 % Bis-Tris Gel
	Running Buffer	1X The NuPAGE® MES SDS Running Buffer
	Transfer Buffer	NuPAGE Transfer Buffer
	Conditions	Running at 180 V for 40 min, wet transfer at 90 V for 90 min
MIF (12.3 kDa)	Gel Type	NuPAGE Novex 12 % Bis-Tris Gel
	Running Buffer	1X The NuPAGE® MES SDS Running Buffer
	Transfer Buffer	NuPAGE Transfer Buffer
	Conditions	Running at 180 V for 40 min, wet transfer at 90 V for 90 min

Table 2.12 Dilutions and duration of incubation for antibodies used in immunoblotting

Antibodies	Dilutions	Incubations
Topol α mouse anti-human (monoclonal)	1:500	Overnight at 4 °C
Goat anti-mouse (polyclonal)	1:10000	1 hr at RT
NG2 rabbit anti-human (polyclonal)	1:750	Overnight at 4 °C
Goat anti-rabbit (polyclonal)	1:3000	1 hr at RT
MGMT mouse anti-human (monoclonal)	1:500	Overnight at 4 °C
Goat anti-mouse (polyclonal)	1:2000	1 hr at RT
S100A8 sheep anti-human (polyclonal)	1:200	1 hr at RT
Rabbit anti-sheep (Polyclonal)	1:2000	1 hr at RT
DEFA3 goat anti-human (polyclonal)	1:160	Overnight at 4 °C
Rabbit anti-goat (Polyclonal)	1:2000	1 hr at Rt
MIF goat anti-human (polyclonal)	1:200	1 hr at RT
Rabbit anti-goat (polyclonal)	1:2000	1 hr at RT
α -tubulin	1:50000	Overnight at 4 °C
	1:2000	1 hr at RT
Goat anti-mouse (Polyclonal)	1:2000	1 hr at RT

2.6 IMMUNOHISTOCHEMICAL STAINING METHODS

2.6.1 Cell Block Preparation from Cell Lines

Cell pellet ($5-10 \times 10^6$) was prepared by washing monolayer cell culture three times with ice-cold PBS. Cells were trypsinised, collected and centrifuged as described in Section 2.2.1. The solid pellet was formed in the conical bottom of 15 ml centrifuge tube. In the meantime, a small aliquot of 1.5% agar (see Appendix B) was heated in a microwave then allowed the temperature drop to 50 °C. A small amount of the liquefied 1.5% agar was dispensed onto the pellet in centrifuge tube until the pellet was covered well. Cell pellet was re-suspended in 1.5% agar solution using a transfer pipette to ensure the cells

were adequately and thoroughly immersed in agar. The solution was then quickly centrifuged at 500 x g for 1 minute and allowed to solidify for 10 minutes at 4 °C. The agar/cell pellet was sandwiched between two biopsy pads (Lomb Scientific, NSW, Australia) and inserted into a histology cassette (Lomb Scientific, NSW, Australia). For the fixation of cells, the cassette was immersed in 10% neutral buffered formalin (Lomb Scientific, NSW, Australia) for 24 hr. The samples were treated as a standard specimen; sections were cut and standard immunohistochemical staining procedure was followed.

2.6.2 Immunohistochemical Staining of Cell Blocks

Immunohistochemistry was performed by Ms Sanaz Maleki, slides containing stained sections were examined by Ms Maleki and confirmed by a qualified pathologist (Dr Janice Brewer and Dr Michael Buckland). Four-µm thick formalin fixed paraffin sections were cut and mounted on SuperFrost Ultra Plus slides. The sections were deparaffinised in xylene (2x5 min) and rehydrated through an ethanol series (100%, 2x3 min and 95%, 1 min) to distilled water. Epitope retrieval was performed at high temperature (95-99 °C) for 20 minutes in either citrate buffer (pH 6.0) or Tris/EDTA buffer (pH 9.0) (Dako, Glostrup, Denmark). The sections were then cooled to RT for 20 minutes and rinsed carefully with distilled water. The sections were then placed in 3% hydrogen peroxide for 15 minutes at RT to block endogenous peroxide, followed by 5 minutes gentle wash in distilled water and immersion in PBS buffer (Amresco, Solon, OH, USA). Antigen retrieval of the sections was performed by treatment with enzyme digestion in Proteinase K (Dako, Glostrup, Denmark) for 10 minutes at 37°C and followed by PBS wash for 5 minutes. To reduce non-specific staining, sections were incubated with Biotin Blocking System (Dako, Glostrup, Denmark) containing biotin and avidin solutions for 10 minutes for each solution at RT. Following the blocking step, primary antibody, TopoIIα monoclonal mouse clone Ki-S1 (1:100 dilution; Dako,

Glostrup, Denmark) was applied for 2 hours at RT, then the complex was detected using either LSABTM+ Universal (Dako, Glostrup, Denmark) or EnvisionTM+ Dual Link Detection Kits (Dako, Glostrup, Denmark). A corresponding dilution of normal fraction IgG (Dako, Glostrup, Denmark) from the species of the animal in which the primary antibody was raised was added to the negative control sections to detect non-specific staining in samples. Chromogenic visualization was accomplished through the use of liquid 3,3-diaminobenzidine (DAB+) substrate (Dako, Glostrup, Denmark) for 10 minutes at RT. All immunohistochemical staining was performed using an automated platform, Dako autostainer (Dako, Glostrup, Denmark). One section from each sample was stained with Harris Haematoxylin (Thermo Fisher, NSW, Australia) and eosin (Thermo Fisher, NSW, Australia) to facilitate examination of general morphological detail of tissue samples. Sections were then dehydrated through graded ethanol into xylene, mounted with coverslips and analysed by light microscopy.

2.6.3 Scoring System for Immunohistochemical Staining of Cell Blocks

Immunostained slides were scored using semiquantitative method, Allred scoring system which estimates the proportion of positively stained cells (scale of proportion score, 0-5) and their staining intensity (scale of intensity score, 0-3) (see Figure 2.4) (Allred et al., 1998). The proportion and intensity scores were combined to produce a final total score. A total score of 0-2 was considered as negative whereas positive staining was corresponded to a total score of 3-8 (detailed in Table 2.13) (Allred et al., 1998). Figure 2.4 summarises the Allred methodology.

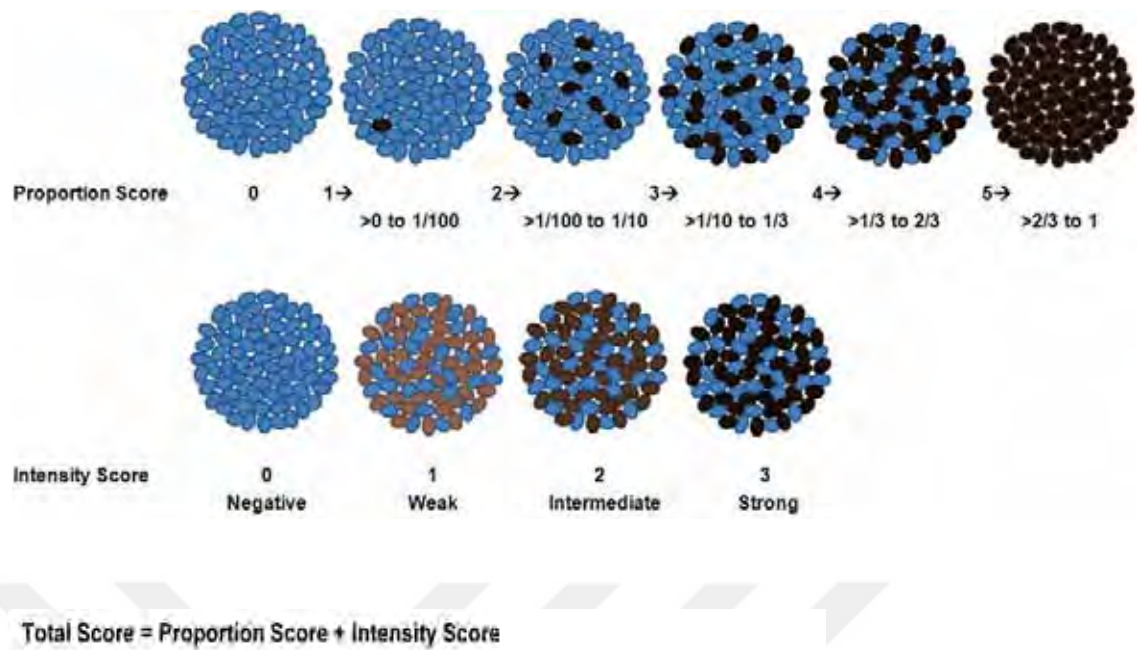


Figure 2.4 Allred scoring system used in assessing staining results

This figure was reproduced from ‘Prognostic and predictive factors in breast cancer by immunohistochemical analysis’ (Allred et al., 1998).

Table 2.13 Allred scoring system used in assessing staining results

Proportion Score	Intensity Score	Total Score	Staining Result
0	0	0	Negative Staining
1	1	2	Weak Staining
1	2-3	3-4	Intermediate to Strong Staining
2-5	1-3	3-8	Weak to Strong Staining

CHAPTER 3

THE ROLE OF TopoII α IN THE CELLULAR RESPONSES TO ETOPOSIDE

3.1 INTRODUCTION

Genetic abnormalities and aberrant protein expression are important hallmarks that characterise glioma. Targeted therapies are used to specifically target molecular pathways that play roles in progression of tumour. Etoposide is one of the traditional antineoplastic agents that targets Topoisomerase II alpha (TopoII α) which is intensely expressed in replicating cells. Variable expression of TopoII α gene has been reported in glioma suggesting that expression of TopoII α could be a predictive marker of patient response in etoposide treatment.

There are numerous studies that focused on the genetic alterations and their effects on the survival time of glioma patients (Ohgaki et al., 2004). TopoII α is present in all phases of the cell cycle as a direct marker of cell-cycle turnover. Expression of TopoII α seems to be associated with the TopoII-related multi drug resistance mechanism. As

previously mentioned, TopoII poisons are included in chemotherapeutic agents that cause TopoII α -associated drug resistance stabilising the TopoII α in cleavage complexes. These complexes become permanent DNA strand breaks and initiate the activity of apoptotic pathways (Bredel et al., 2002a).

Over the years, interactions between TopoII α and etoposide have grown to be an important part of a network of resistance mechanisms which involve the contribution of various other drug resistance-related components. Therefore it is necessary to investigate and elucidate the relationship between TopoII α and etoposide in glioma in order to establish a clear understanding of chemoresistance.

Working Hypotheses of this chapter:

1. Glioma cell lines with mutant *p53* gene (T98G, M059K, M059J) accumulate high levels of TopoII α in contrast to cell lines carrying wild type *p53* (A172, U-87 MG).
2. Cell lines with elevated TopoII α expression indicate relatively high sensitivity to etoposide.

The overall aim of this chapter is to examine the cytotoxic effect of etoposide on a panel of glioma cell lines and relate the response to the levels of TopoII α expression. Moreover, the primary objective was to assess the capacity of TopoII α expression as a discriminator of etoposide response.

The specific aims of this chapter of work were to:

1. Measure the endogenous protein and mRNA expression levels of TopoII α in *p53* wild type and mutant glioma cell lines using immunoblotting and qRT-PCR.
2. Perform a dose-response curve by treating cells with different concentrations of etoposide and assessing viability using the MTS cell proliferation assay. IC₅₀ (or IC₅₀s) will be determined for each etoposide treated cell line.
3. Determine if etoposide directly degrades protein expression levels of TopoII α by measuring the protein expression pre- and post-treatment of etoposide.

3.2 METHODS

3.2.1 Characteristics of Glioma Cell Lines

The immortalised human glioma cell lines, T98G, M059K and M059J bearing mutant *p53* gene and A172 and U-87 MG bearing wild type *p53* were purchased from the American Type Culture Collection (ATCC). All cell lines were all derived from *de novo* malignant glioma tumours. *PTEN* mutation and *p14ARF/p16* deletion have also been identified in T98G, A172 and U-87 MG cell lines (Ishii et al., 1999). Presence of loss of heterozygosity at *PTEN* locus have been reported in 74% of gliomas, also *PTEN* mutations in both alleles which is associated with tumour development have found in 44% of glioma samples (Wang et al., 1997b). Another study evidenced biallelic inactivation of *PTEN* gene with mutations found to be present in 28% of glioma samples (Zhou et al., 1999).

T98G cell line was derived from human glioma tumour tissue of a 61-year old male Caucasian. In 1979, Stein reported no tumourigenicity of T98G cells when inoculated in nude mice both subcutaneously and intra-cerebrally (Stein, 1979). Anchorage independence of cells was known as proliferation ability of cells without an attachment to extracellular matrix proteins and associated with being tumorigenic when inoculated in animals (Freedman and Shin, 1974). However, work by Stein showed that anchorage independence of T98G cells was not sufficient to form tumours in animals. In addition to their immortality and anchorage independency, T98G cells become arrested in G0/G1 under stationary phase conditions (high density of cells or low serum concentration) similar to normal cells. (Stein, 1979).

A172 (Giard et al., 1973) and U-87 MG cells were derived from tumour tissues of 53 years old male and 44 years old female, respectively. While U-87 MG cells were highly

tumorigenic in immunocompromised mice when inoculated subcutaneously, no tumour progression by A172 cells has been reported (Ishii et al., 1999).

M059K and M059J cell lines were established from the different regions of the same tumour tissue from 33 years old male. M059J cells were found to be relatively sensitive to radiation than M059K cells. Whereas increased sensitiveness to chemotherapeutic agents was reported for M059K cells (Allalunis-turner et al., 1993). In the light of such data, studies investigating the reasons of hypersensitivity of M059J cells to ionising radiation identified that DNA repair mechanisms were involved in this process. DNA-dependent protein kinase (DNA-PK) is an enzyme that plays an important role in the repair of double strand breaks. M059J cells fail to express the catalytic subunit of DNA-PK which results in an increased radiosensitivity. However, M059K cells express intact DNA-PK protein that causes a relatively radioresistant phenotype (Lees-Miller et al., 1995). The characteristics of these cell lines are summarised in Table 3.1.

Table 3.1 Characteristics of glioma cell lines

Cell Line Description	T98G	A172	M059K	U-87 MG	M059J
Diagnosis	Glioblastoms	Glioblastoma	Glioblastoma	Glioblastoma	Glioblastoma
Morphology	Fibroblast	Astrocytic	Fibroblast	Epithelial	Fibroblast
Donor's Gender	Male	Male	Male	Female	Male
Donor's Age	61 years	53 years	33 years	44 years	33 years
Tumourigenicity in Nude Mice	No	No	Yes	Yes	Yes
Molecular Characteritics	p53-mt	p53-wt	p53-mt	p53-wt	p53-mt
	PTEN-mt	PTEN-mt	Intact DNA-PK	PTEN-mt	Deficient DNA-PK
	P14ARF/p16*	P14ARF/p16*	chemosensitive	P14ARF/p16*	Radiosensitive

Abbreviations: *: deletion, mt: mutation, wt: wild type gene

3.2.2 Determining Optimal Cell Number for 96-Well Plating

Tissue culture maintenance of the glioma cell lines was performed as previously described in Section 2.2.1, Material and Methods, Chapter 2. A testing range of cell numbers from 1000 to 50000 per ml was prepared and 100 µl of cell mixture was dispensed into each well of flat-bottomed 96-well plate. Six wells for each cell density were assayed during a 72 hr time course (at 24, 48 and 72 hr) via MTS assay as explained in Section 2.2.3, Material and Methods, Chapter 2. Absorbance of formazan product directly related to the number of living cells was measured at 490nm. Graphs in Figure 3.1 indicate observed absorbance versus number of cells per ml monitored for 72 hr. Optimum cell densities were established by selecting absorbance values near 1.0 at 72 hr which corresponded with a cell density on x-axis. While values near 1.0 reproduce exponential growth phase, values greater than 1.0 reflect reduced proliferation which is followed by a linear and subsequently by a plateau growth phase.

3.2.3 Preparation of Etoposide

Etopophos® (etoposide phosphate) (Bristol-Myers Squibb Co., New York, NY, USA) is a lyophilised and water-soluble powder form of etoposide (VP-16-213). Each Etopophos® vial contains 113.6 mg of etopophos which is equivalent to 100 mg of etoposide. Etopophos powder was dissolved by adding 11.32 ml of Ca^{2+} and Mg^{2+} free 1X phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA) into the vial to reach the final concentration of 15 mM etoposide as stock solution. The solution was stored at 4 °C.

3.2.4 Experimental Design for Etoposide Treatment

The MTS assay was used to monitor etoposide toxicity on glioma cell lines as described in Section 2.2.3, Material and Methods, Chapter 2. A wide range of etoposide dilutions

(0.78-200 μ M) were prepared for initial experiments. Drug dilutions were applied to glioma cell cultures in 96-well plates (Thermo Scientific, Waltham, MA, USA). A blank with only media and a control cell group without etoposide treatment was included in each plate.

3.2.5. Determining Efficiency of TopoII α Gene Expression Using RT-qPCR

RT-qPCR was performed on cDNA from T98G, A172, M059K, U-87 MG and M059J (see Section 2.4.4, Material and Methods, Chapter 2). To amplify TopoII α , the Taqman gene expression assay Hs00172214_m1 (TOP2A) (Applied Biosystems, Foster City, CA, USA) was used and RNA quantity was expressed relative to Eukaryotic 18S rRNA Endogenous Control (VIC/MGB Probe, Applied Biosystems, Mulgrave, VIC, Australia). The efficiency of qRT-PCR reactions for both TopoII α and 18S were between 100% and 110%.

3.2.6 Statistical Analysis

Statistical analysis (Student's t-test) of the data was performed using GraphPad Prism software (v.4, GraphPad Software, Inc.). Asterisk rating system (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001) were used when representing significance.

3.3 RESULTS

3.3.1 Determining Optimal Cell Number for 96-Well Plating

Optimal cell number for 96-well plating was determined to achieve ideal cell attachment and growth per well. Established cell numbers were further used in cytotoxicity assays performed by MTS assay as explained in Section 2.2.4, Material and Methods, Chapter 2. The optimal cell densities for the cell lines; T98G, A172, M059K, U-87 MG and M059J were determined and illustrated in Figure 3.1. These results demonstrated the optimal plating density for T98G, A172, M059K, U-87 MG and M059J cells in 96-well plates to be 15000, 25000, 40000, 25000 and 50000/ml, respectively (see Table 3.2). M059K and M059J cell lines required a higher initial cell seeding number compared to the cell numbers optimised for T98G, A172 and U-87 MG cell lines to achieve efficient growth condition. Each well was filled with 100 μ l of cell suspensions which represents the actual number of cells per well.

Table 3.2 Optimum number of cells to use for cytotoxicity assays

Cell Lines	Cells/ml	Cells/well
T98G	15000	1500
A172	25000	2500
M059K	40000	4000
U-87 MG	25000	2500
M059J	50000	5000

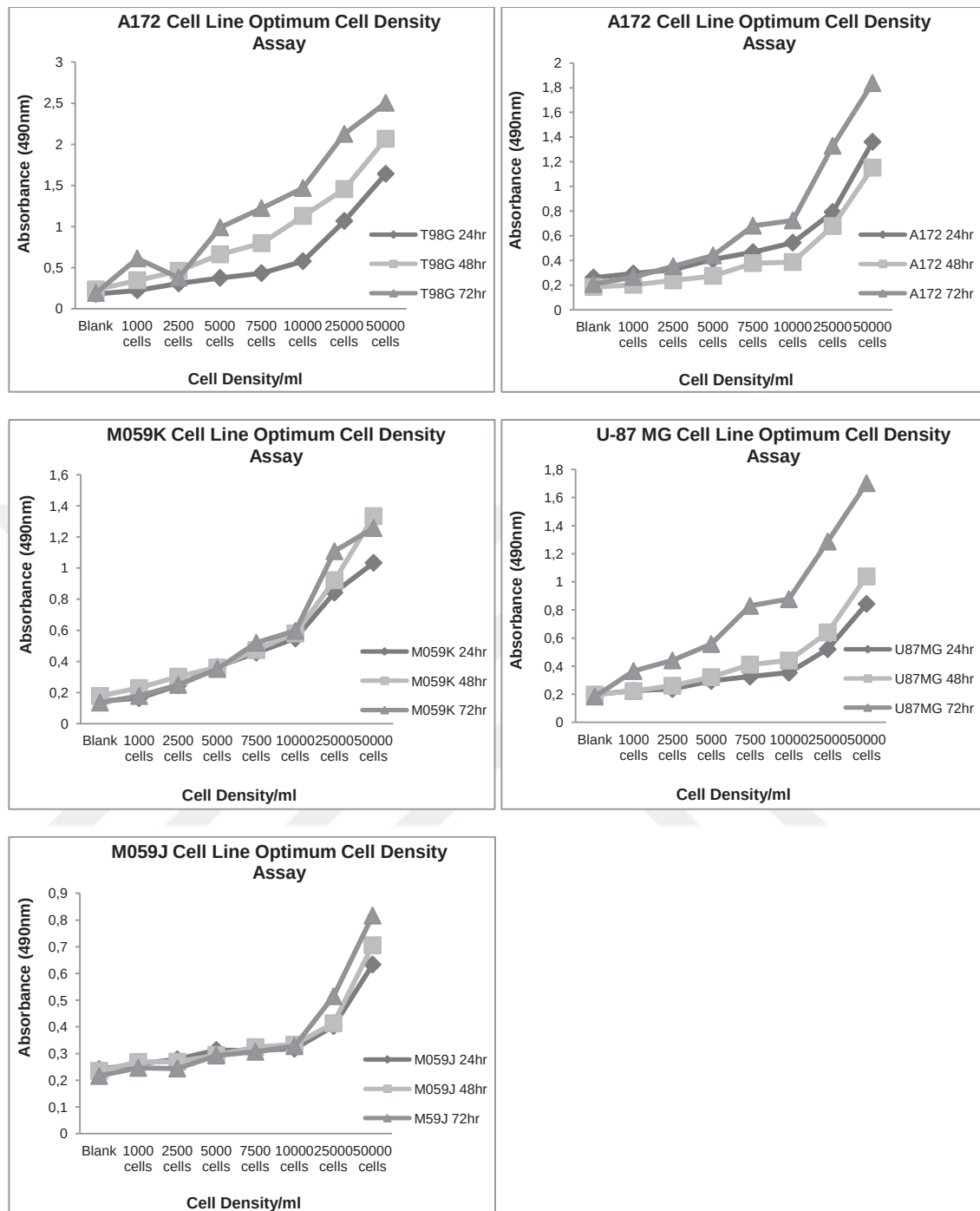


Figure 3.1 Results of optimum cell number assay at 24, 48, 72 hr

Graphical representation of the glioma cell line proliferation measured at three time points (24, 48 and 72 hr) with the MTS assay. Five glioma cell lines at concentrations ranging from 1000 to 50000 cells were plated in 96 well-plates.

3.3.2 Establishing Endogenous Levels of TopoII α in Glioma Cell Lines

Endogenous expression levels of TopoII α were measured in five glioma cell lines using immunoblotting, IHC and qRT-PCR. Variable levels of TopoII α were observed in cell lines. *p53*-mt cell lines M059K and M059J showed elevated expression of TopoII α at a level greater than 6-fold higher than A172 and U-87 MG harbouring *p53*-wt gene. Even though T98G is a *p53*-mt cell line a moderate TopoII α expression was measured in total protein using immunoblotting (see Figure 3.2).

Figure 3.3 demonstrates total TopoII α protein expression levels detected by immunohistochemistry (IHC). All cell lines were positive expressors of TopoII α . Neither A172 (20%) nor U-87 MG (20%) stained as strong as M059K (98%) and M059J (75%). T98G showed again a moderate degree of expression with a 45% staining (see Figure 3.3).

Expression of TopoII α was calculated relative to Human Brain Reference RNA (NBTot). The results confirmed significantly higher ($p < 0.05$, unpaired t-test) mRNA levels in T98G, M059K and M059J cell lines than U-87 MG. No significant change in mRNA expression was found between A172 and U-87 MG. In contrast to these findings, levels of TopoII α mRNA expression in human brain reference samples namely NBPCS, NBPCP and NBORF was reasonably low as anticipated for normally functioning cells (see Figure 3.4). These results are consistent with what was quantified by immunoblotting and IHC staining.

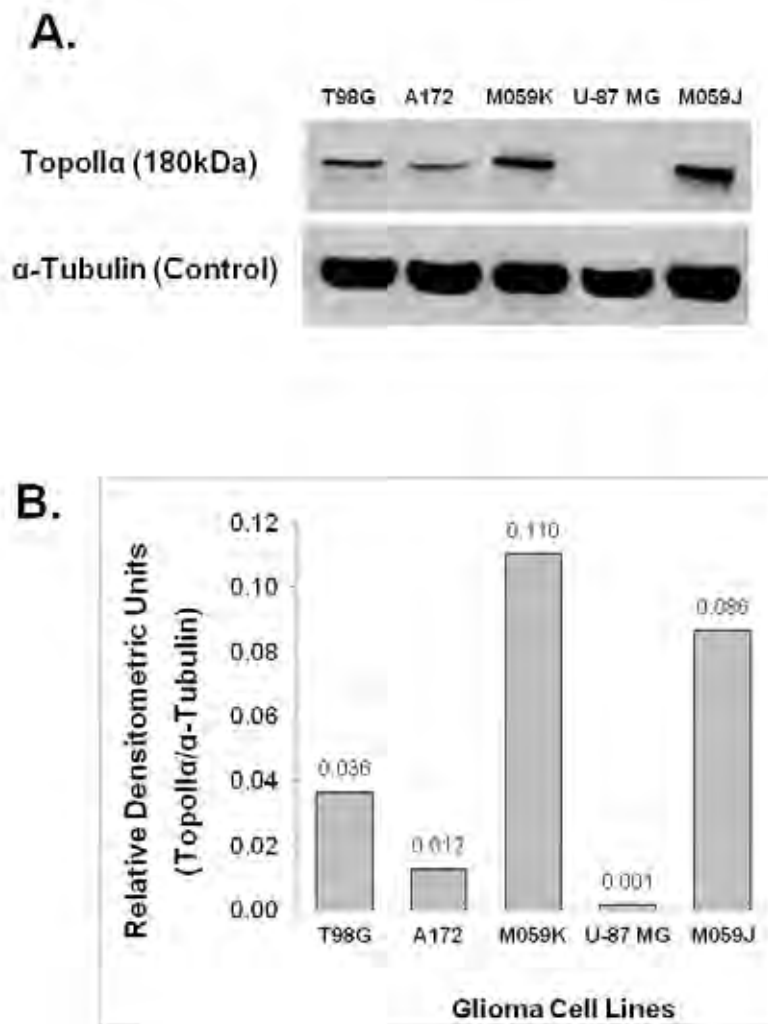


Figure 3.2 Endogenous protein expression levels in a panel of five glioma cell lines

(A) Representative immunoblotting. Protein extracts of the five glioma cell lines were assessed with immunoblotting using a specific antibody against TopoIIα. **(B)** Densitometric analysis. The y-axis represents arbitrary densitometric ratios.

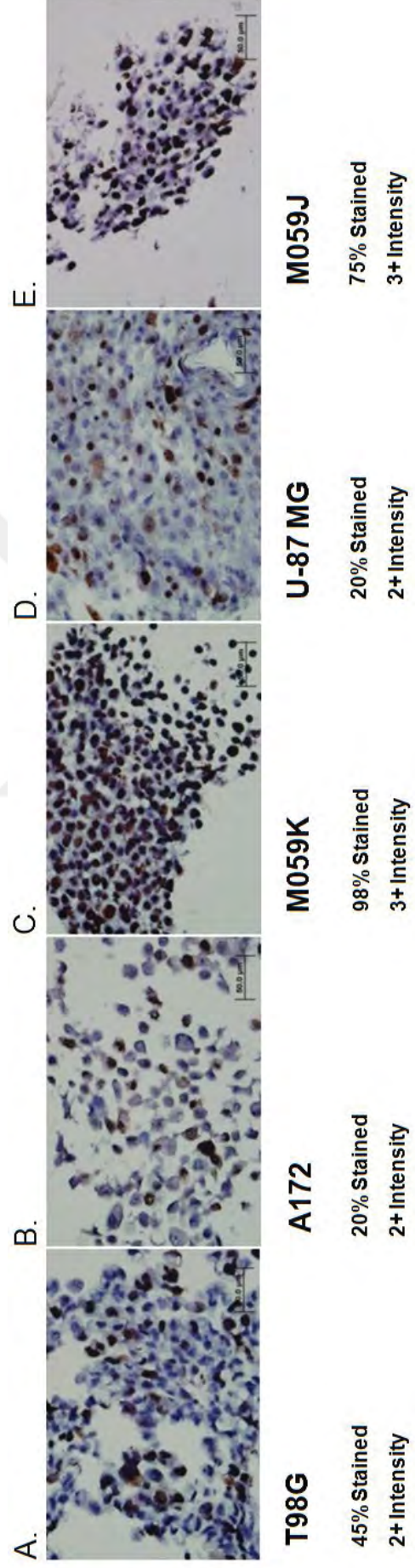


Figure 3.3 Immunohistochemical staining of glioma cell blocks for TopoIIα

IHC analysis with monoclonal mouse antibody for detecting TopoIIα expression in the five glioma cell lines; **(A)** T98G, **(B)** A172, **(C)** M059K, **(D)** U-87 MG and **(E)** M059J. Magnification bar represents 50μm in all images.

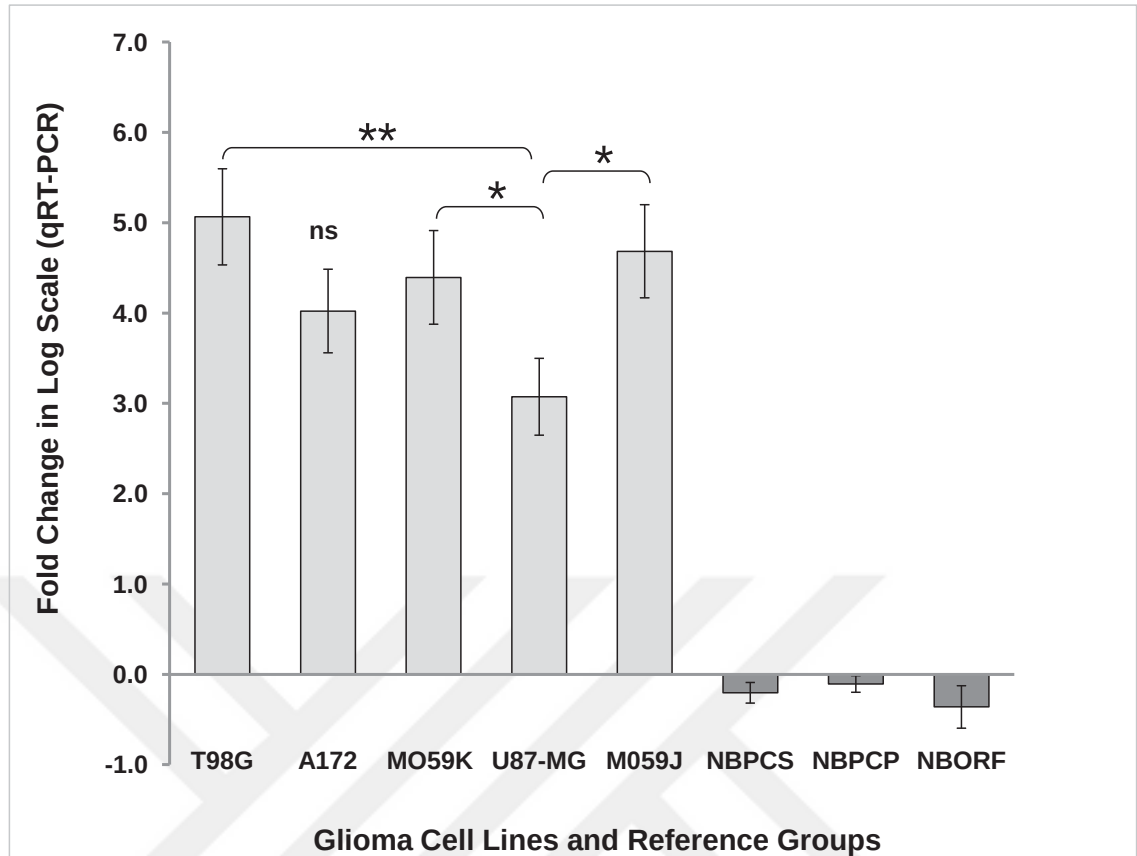


Figure 3.4 Relative mRNA expression levels measured by qRT-PCR

Each column represents the relative expression level in log scale in relation to the mRNA expression of human brain reference RNA (NBTot). Unpaired t-test was used. *, $p < 0.05$; **, $p < 0.01$; ns, non-significant as compared with U-87 MG cell line (p53-wt).

3.3.3 Response Characteristics of Glioma Cell Lines to Etoposide Treatment

The IC₅₀ was determined for five glioma cell lines at 24, 48 and 72 hr following etoposide treatment. Dose-response plots shown in Figure 3.5 were created to determine the appropriate drug concentration range and the optimum time-point to establish IC₅₀ doses for each cell line. Cells were exposed to 0.78 μ M to 200 μ M of etoposide. A significant decrease in the cell growth was observed at 48 hr and IC₅₀ dose was reached for all cell lines. T98G remained still the most resistant to etoposide. After 72 hr exposure to etoposide, a considerable reduction in cell viability was detected in the wells treated with ≥ 25 μ M etoposide and 200 μ M treatment resulted in complete cell death. Based on the results from Figure 3.5, different dose ranges were determined for relatively more sensitive cell lines, M059K and M059J from 0.19 to 50 μ M and more resistant cell lines T98G, A172, U-87 MG from 0.78 to 200 μ M to establish IC₅₀ doses of cell lines. Cell lines were grouped as resistant (T98G), moderate (A172, U-87 MG, M059J) and sensitive (M059K) depending on their responses to etoposide (see Figure 3.6). IC₅₀ doses were calculated at 48 hr post-treatment with etoposide shown in Table 3.3. Different responses to etoposide were observed among all glioma cell lines. T98G cell line showed the highest IC₅₀ value, 17.27 μ M and was found to be significantly more resistant to etoposide than other glioma cell lines. IC₅₀ doses of A172, M059K, U-87 MG and M059J were determined to range between 0.53 to 1.95 μ M. A significant difference between M059K and M059J was also observed in response to etoposide treatment. Unexpectedly, T98G cell line was also identified as a high expressor of TopoII α yet exhibited substantial level ($p < 0.05$) of resistance to the drug. Variable TopoII α expression levels were measured in the moderately responsive cell lines, A172, U-87 MG and M059J. This series of experiments highlighted variable

responses to etoposide treatment which was observed irrespective of the expression patterns of TopoII α .

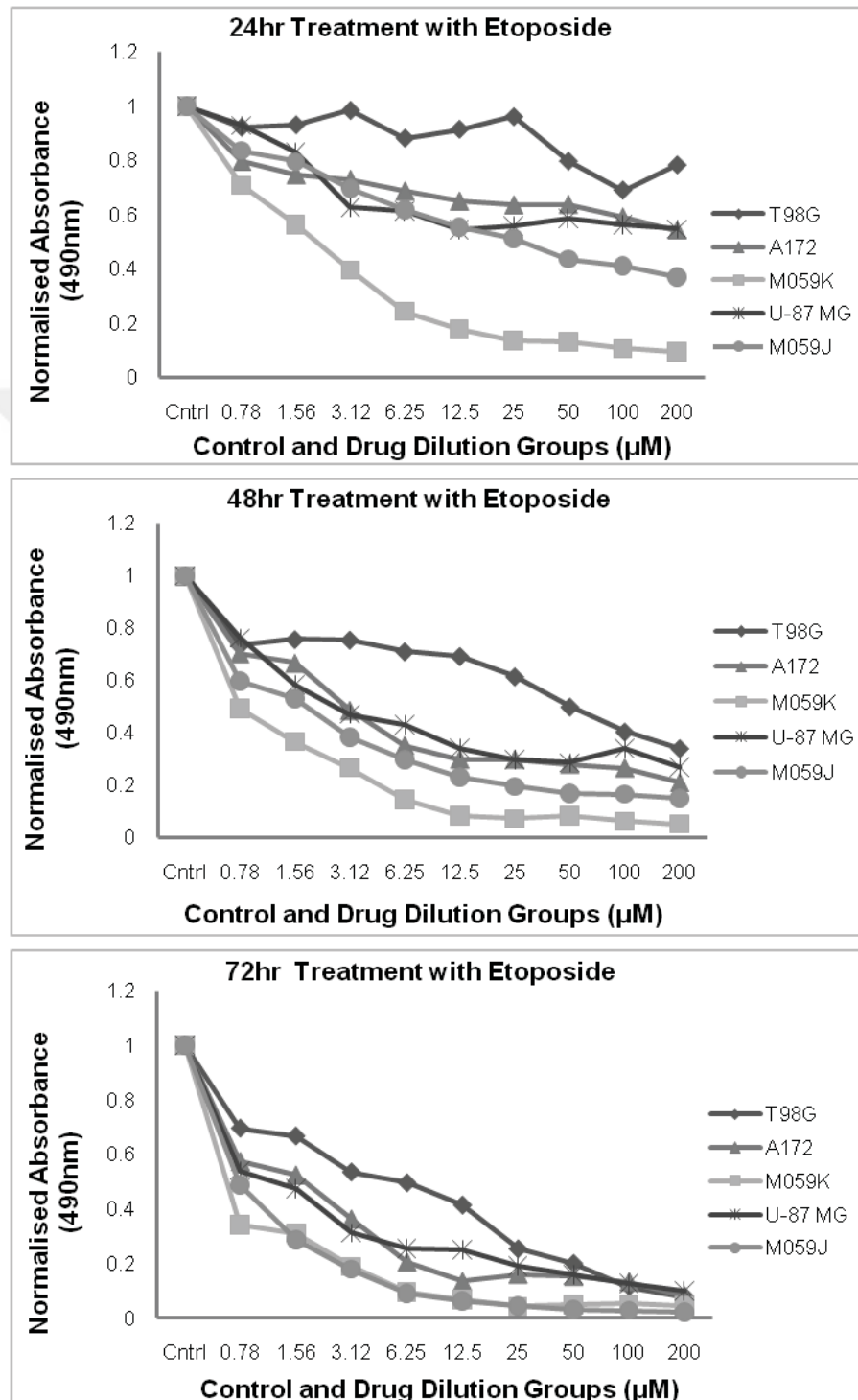


Figure 3.5 Response of glioma cell lines to etoposide treatment (24, 48 and 72 hr)

Graphs represent viability of cells at 24, 48 and 72 hr following etoposide treatment. Measurements were collected using MTS assay.

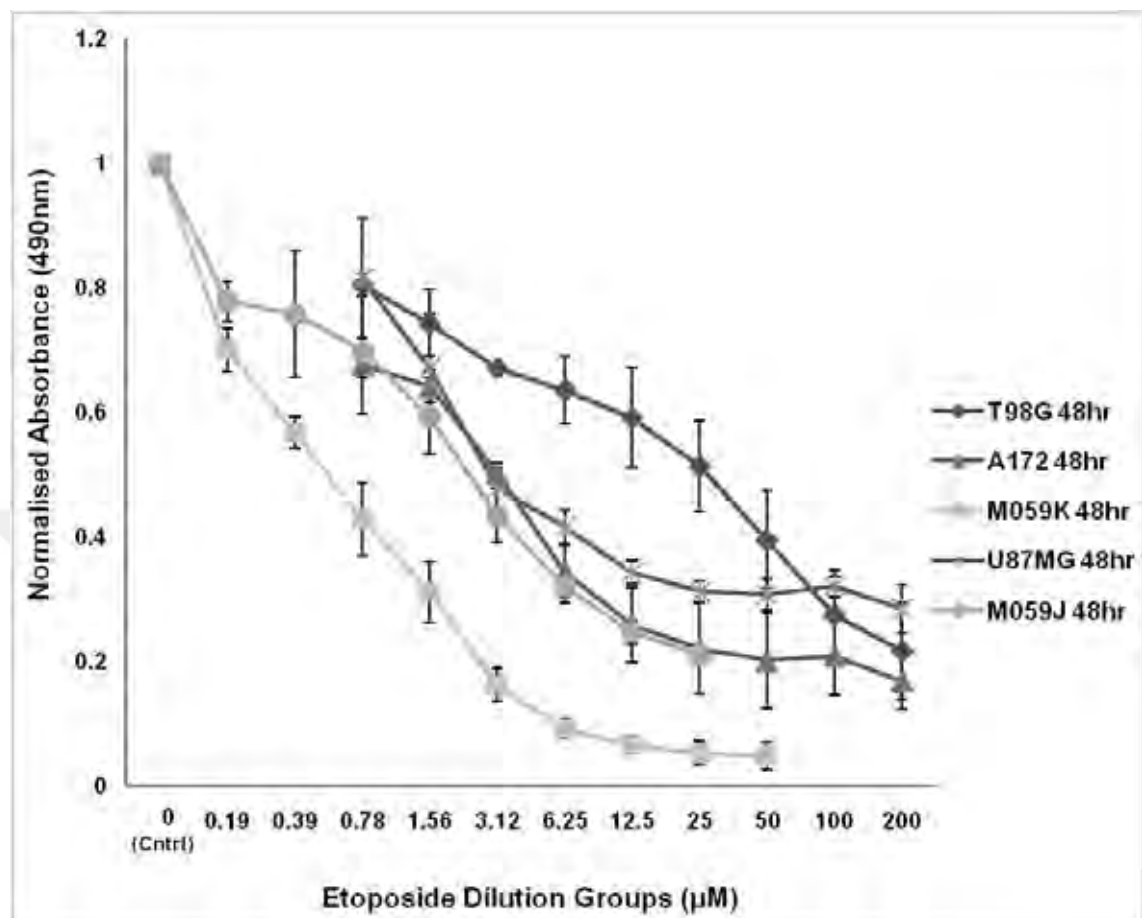


Figure 3.6 Combination of response results of cell lines treated with different dose ranges of etoposide for 48 hr

Data are presented as the mean of three independent experiments ($n = 3$) for each cell line at eleven different etoposide concentrations. Error bars indicate mean $\pm$ standard deviation. Dose-response curves were drawn by plotting the absorbance at 490nm and expressed as the values calculated relative to the untreated control, against drug concentrations examined (0.19-200 μ M).

Table 3.3 IC₅₀ doses (μM) of glioma cell lines at 48 hr post-treatment

	T98G	A172	M059K	U-87 MG	M059J
IC ₅₀ doses of etoposide (μM)	17.27 ± 5.33	1.95 ± 0.35	0.53 ± 0.10	1.58 ± 0.30	1.68 ± 0.69
T98G	-	**	***	**	**
A172	**	-	**	ns	ns
M059K	**	**	-	**	*
U-87 MG	**	ns	**	-	ns
M059J	**	ns	=	ns	-

The concentration of etoposide required to cause a loss in viability by 50% (IC₅₀) for each cell line was calculated from the dose-response curves shown in Figure 3.6.

*; $p < 0.05$ indicates significant difference between IC₅₀ doses of cell lines when compared with the most resistant cell line T98G. **; $p < 0.01$ shows significance between IC₅₀ doses of M059K and M059J cell lines. ***; $p < 0.001$ indicates significance of difference between IC₅₀ doses of M059K, and U-87 MG and A172. ns; non-significant, indicates no significant association (Student's t-test was performed)

3.3.4 Alterations in the Total Protein and mRNA Levels of TopoII α Post Etoposide Treatment

To determine if there was a direct effect of etoposide on TopoII α expression as a result of DSBs and subsequent degradation, protein and RNA were collected before and after etoposide treatment. Glioma cell lines were exposed to the IC₅₀ dose of etoposide for 48 hr. Levels of TopoII α in T98G cells were found to be strongly induced after etoposide treatment, while no considerable increase was observed in M059K cells (see Figure 3.7). A172 cells indicated slightly decreased protein levels of TopoII α , however elevated expression of protein was observed in U-87 MG and M059J cell lines. Gene expression of TopoII α at translational level was not consistent with the pattern of expression at the level of transcription.

While elevated levels of TopoII α protein in T98G cells were detected by immunoblotting after treatment with etoposide, no considerable change in mRNA expression was found by qRT-PCR (see Figure 3.8). Interestingly, this cell line was also characterised with its highest degree of resistance to etoposide (shown in Section 3.3.3). Immunoblotting results demonstrated slightly increased protein levels in M059K which was the most sensitive cell line to etoposide. In addition to this, there was no change in mRNA levels pre and post-treatment. Other cell lines showed either small increase (U-87 MG and M059J) or decrease (A172) in protein levels, while mRNA levels were found notably low in all these three cell lines. In addition to these results, low basal level mRNA expression of TopoII α was confirmed in human brain reference samples.

Five glioma cell lines showed variable levels of protein expression after 48 hr continuous drug treatment. No clear association was established between protein expression and response characteristics of the cell lines to etoposide treatment. Our initial hypothesis suggested that drug induced-degradation of TopoII α results in reduced levels of TopoII α following etoposide treatment. However, these results confirmed that it is not essential to observe decreased levels of TopoII α post-treatment, to monitor response of cell lines to etoposide. Therefore, it is concluded that TopoII α expression levels alone did not exhibit a substantial performance in predicting etoposide response of the glioma cell lines.

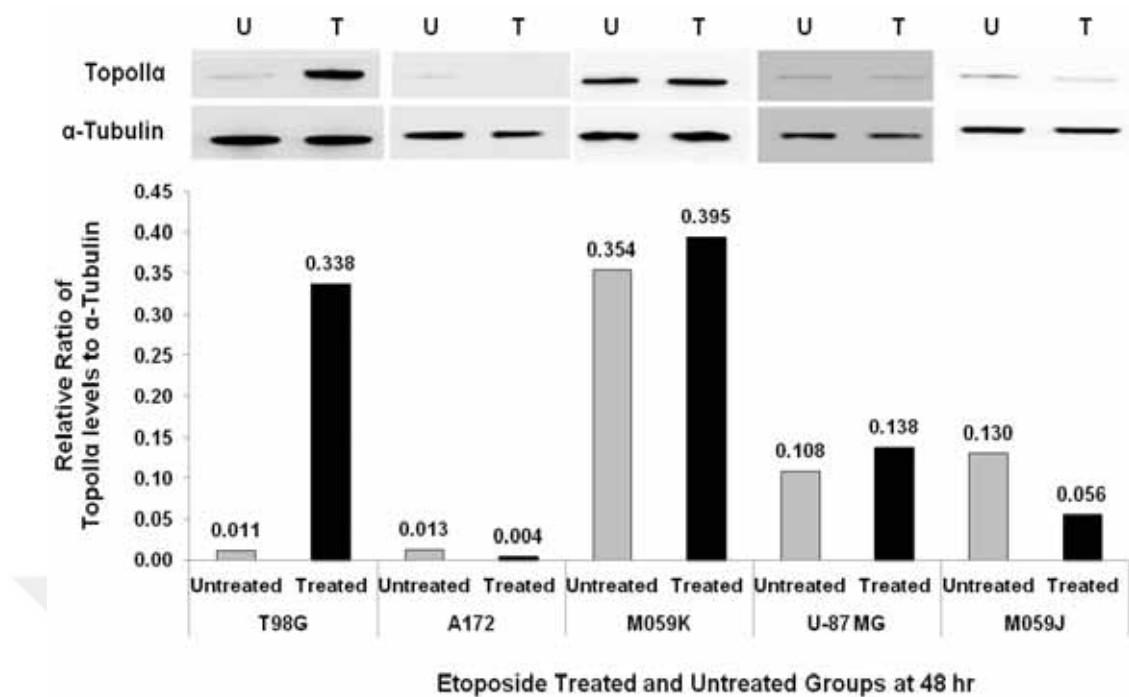


Figure 3.7 Levels of TopoIIα pre and post etoposide treatment

Representative Immunoblotting (**top**). Immunoblots of the five glioma cell line-derived protein extracts were probed with a specific antibody against TopoIIα protein. Quantitative immunoblot analysis (**bottom**). The y-axis represents arbitrary densitometric units.

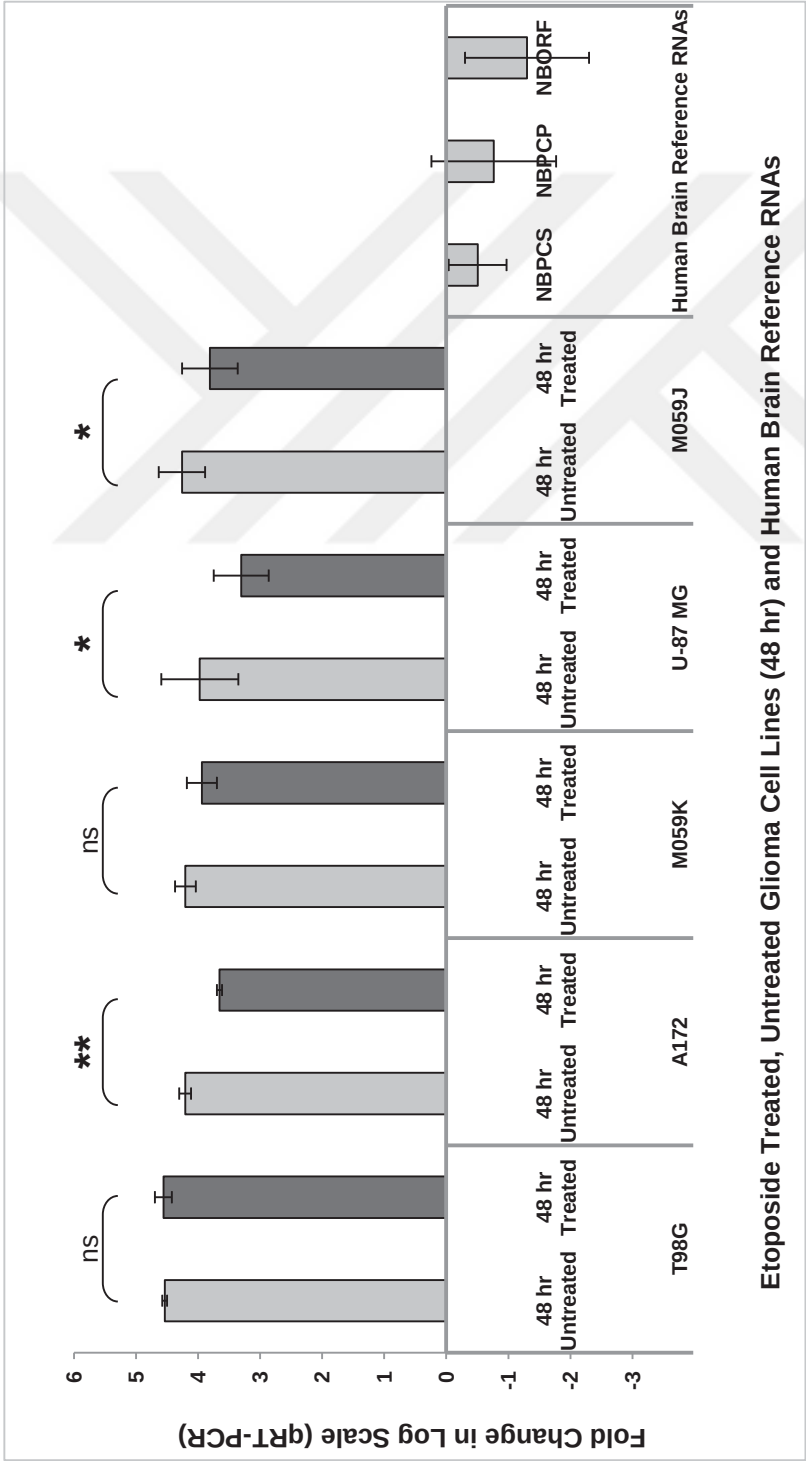


Figure 3.8 Relative mRNA expression levels measured by qRT-PCR

Each column represents the relative expression level in log scale in relation to the mRNA expression of human brain reference RNA (NBTot). *, $p < 0.05$; **, $p < 0.01$; ns, non-significant as compared with untreated group for each cell line.

3.4 DISCUSSION

The main objective of this chapter was to examine expression levels of TopoII α in glioma cell lines in order to establish a link between the amount of TopoII α in cells and its efficacy as a marker in predicting etoposide response. Association between the expression levels of TopoII α and response to etoposide was evident however no clear correlation could be established in glioma cell lines, unlike other cancer types. Data from this chapter suggests that other drug resistance-related factors are involved in this single marker targeted therapy.

The range of physiologically relevant concentrations of etoposide has been reported to be 20-50 μ M (Sung et al., 2006). The IC₅₀ concentration of etoposide for the most sensitive cell line, M059K was 0.64 μ M. The T98G cell line was refractory to etoposide with an IC₅₀ dose of 28.85 μ M. Matsumoto and colleagues reported an IC₅₀ dose of 31 μ M for T98G (Matsumoto et al., 1999). Another study reported IC₅₀ concentration of etoposide was 42 μ M and 0.85 μ M for T98G and U-87 MG cells, respectively (Mohri et al., 2000). These findings are in line with the data presented in this chapter. In addition, previous research in our laboratory has demonstrated that siRNA knock-down of TopoII α expression in M059K cells did not establish a complete resistance to etoposide. Transfection of T98G cells with the TopoII α siRNA resulted in no significant effect on cell viability (Sevim et al., 2011).

p53 functions as a regulator of cell cycle and expression of p53-wt has been suggested to be a potent mediator to down-regulate expression from the TopoII α promoter (Sandri et al., 1996; Wang and Harris, 1997). Results of this chapter showed that glioma cell lines M059K and M059J harbouring p53-mt gene exhibited elevated expression of TopoII α and were sensitive to etoposide. Transfection of *TopoII α* into resistant cell lines

sensitised cells to etoposide (Asano et al., 1996; Zhou et al., 2001). Expression levels of TopoII α was found relatively low in A172 and U-87 MG cell lines, which are both *p53*-wt. It is hypothesised that the expression of intact and active *p53* is an upstream regulator of TopoII α . Regarding their response to etoposide, no considerable difference due to these low expression levels was observed when compared with M059K and M059J. This result was unexpected because even though A172 and U-87 MG expressing low levels of TopoII α , their etoposide sensitivity was found similar to potent TopoII α expressors. However, Patra and co-workers suggested an alternative mechanism for the increased sensitivity of *p53*-wt prostate cancer cell line LNCaP to a novel TopoII α inhibitor, MHY336 (Patra et al., 2011). The interaction between MHY336 and DNA gives rise to generation of DSBs through inhibition of TopoII α and induces the up-regulation of *p53* protein which eventually initiates *p53*-dependent apoptotic pathways (Patra et al., 2011). Therefore, active and intact *p53* proteins could mediate the cell death when induced by TopoII α inhibitors. In addition to *p53*-dependent mechanism, other factors may affect the response of cell lines to etoposide. For example, BH3-only protein, BID which is a member of Bcl-2 family has been shown to be essential as an apoptotic mediator upon double strand DNA damage. Ionising radiation and/or etoposide-induced DSBs can trigger apoptotic pathways through activation of BID which potentially functions as a tumour suppressor (Maas et al., 2011).

Interestingly, in contrast to both *p53*-mt and *p53*-wt but sensitive cell lines, T98G (*p53*-wt) indicated very remarkable resistance to etoposide. Although its TopoII α expression levels were moderate, such a response to etoposide was unexpected. Resistant phenotype of T98G cells could be due to Multi Drug Resistance Associated Proteins (MRPs). *MRP* gene has been shown as one of the down-stream effectors of *p53*-wt

which negatively regulates MRP gene transcription (Wang and Beck, 1998). Mohri and colleagues showed 4.5-fold increased expression levels of MRP protein in T98G cell line in comparison with U-87 MG cells (Mohri et al., 2000). In another study, increased etoposide uptake into the cells and enhanced sensitivity to etoposide were observed in T98G cells transfected with MRP2 antisense (Matsumoto et al., 2005). In addition, the relationship between HIF-1 α and MRP1 has also been reported. This was evidenced by the transfection of an siRNA specifically silencing HIF-1 which resulted in decrease in expression levels of *MRP1* gene and consequently increased sensitivity of T98G cells to etoposide and doxorubicin (Chen et al., 2009a). Overexpression of Gli2 protein which is a member of HedgeHog pathway has been correlated with chemoresistance of glioma cells by upregulating MDR related genes; *MDR1*, *MRP1*, *LRP*, *MGMT*, *Bcl-2* and *Survivin* (Cui et al., 2010). TopoII poisons stabilise DNA-TopoII α cleavage complexes and eventually result in a decreased level of TopoII α in the cells due to rapid degradation of TopoII α by degradation mechanisms. (Desai et al., 1997; Mao et al., 2000). However in T98G cells, we did not observe a decrease in the expression levels of TopoII α following etoposide treatment which can be explained by MRP proteins that cause insufficient concentrations of drug in tumour cells (see Figure 3.14, A). To conclude, dramatic change in the protein levels of TopoII α after etoposide exposure might be developed by the roles of MRPs in drug uptake.

Following 48 hr etoposide exposure, a significant decrease in the levels of TopoII α was observed in M059J cells. Rapid degradation of TopoII in TopoII α -DNA-etoposide cleavage complexes by SUMO-1, ubiquitin and 26S dependent pathways were suggested to occur. M059J cells were the only cells representing this degradation process among five glioma cell lines. However, this degradation pathway suggests that bare DSBs in turn trigger DNA repair mechanisms such as TDP1 and subsequently

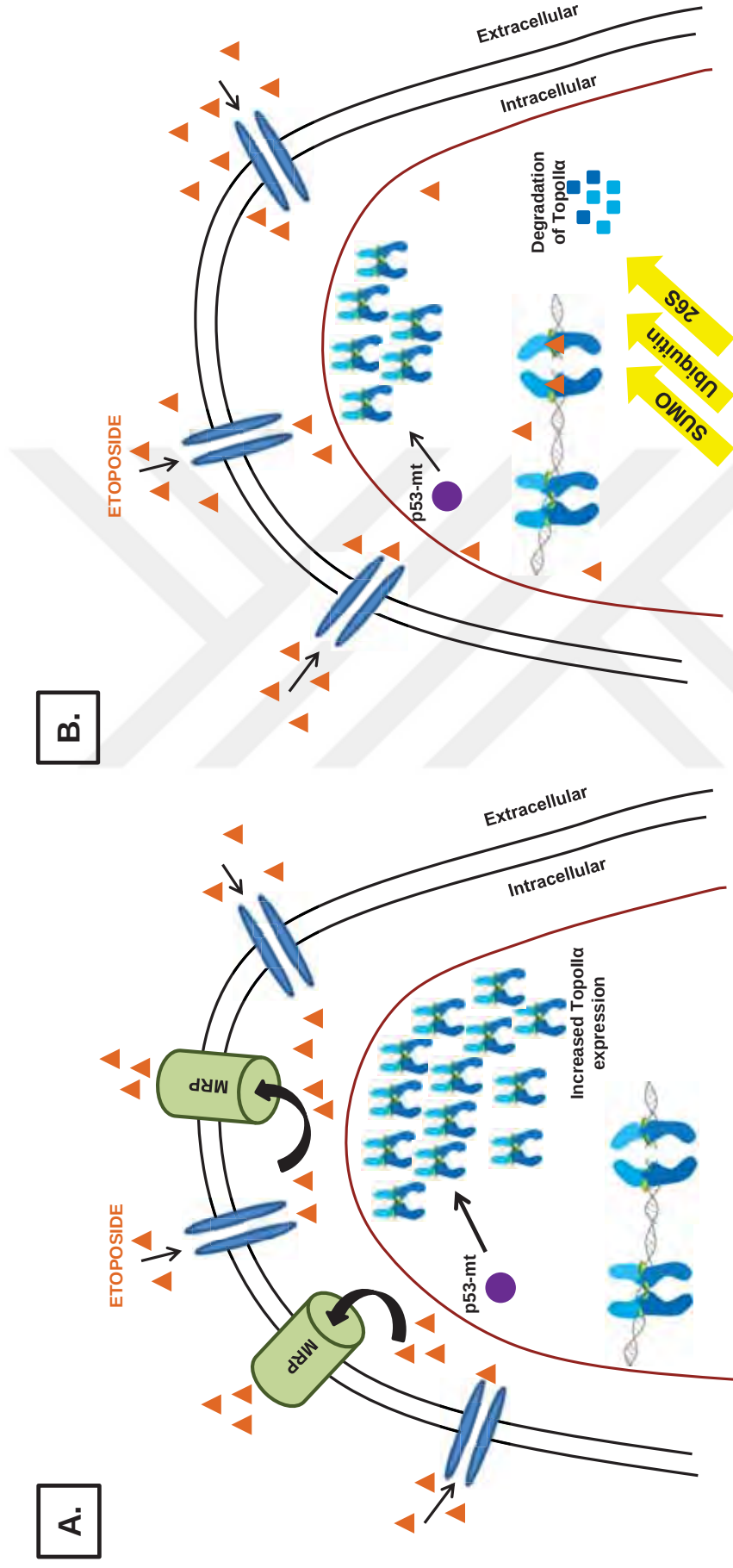


Figure 3.9 Schematic illustration of the proposed mechanisms of etoposide transport in two glioma cell lines

Potential interaction between etoposide and TopoIIα (A) mechanism in T98G cell line involving drug resistance proteins that cause insufficient drug concentration in the cell and (B) mechanism in M059J demonstrating considerable level of etoposide in the cells which might result in decreased levels of TopoIIα because of activated degradation mechanisms. The arrows indicate the direction of transport.

Abbreviations: MRP: multi drug resistance associated proteins, SUMO: small ubiquitin-like modifier protein.

reverse the effect of etoposide (see Figure 3.9, B) (Barthelmes et al., 2004; Desai et al., 1997; Mao et al., 2000). Because of deficient DNA-PK gene in M059J cells, we propose that no considerable level of DSBs were repaired, hence, etoposide exerted its cytotoxic effect more efficiently. Variable levels of the proteins involved in degradation mechanisms could play a role in differential response of glioma cell lines to etoposide.

TopoII α was highly expressed in both M059K and M059J cells. Even though both cell lines were derived from the same tumour, they exhibit differential responses to drug and radiation therapies. Interestingly, M059K cells were slightly more sensitive to etoposide treatment compared to M059J cells. Contribution of DNA-PK to DNA cross-linking and drug resistance has not been clearly identified. However, DNA-PK-independent DNA repair mechanism might be an explanation for the reduced sensitivity of DNA-PK deficient M059J cells. DNA-PK repairs DSBs by non-homologous DNA end joining (NHEJ). In a study, M059K cells treated with a DNA-PK inhibitor, wortmannin showed that complete blockage of DNA-PK dependent NHEJ can be compensated by a DNA-PK independent process rendering slow and abnormal repair process. DNA-PK independent NHEJ mechanism in M059J cells might play an important role in gaining chromosome aberrations (Virsik-Kopp et al., 2003). Fluorescence in situ hybridization analyses demonstrated that M059J cells are near pentaploid with multiple abnormalities and have a much broader distribution of chromosome number when compared with M059K cells. Association between genome instability and malignancies have been reported. (Colnaghi et al., 2011; Nobusawa et al., 2010). In light of these findings, we could conclude that alterations in addition to DNA-PK may exist between M059K and M059J cell lines which could possibly contribute their altered responses to the results observed in this study.

In contrast to the reports suggesting TopoII α as a marker of response, our results are in accordance with a growing body of literature that demonstrates poor predictive potential of TopoII α in reflecting clinical outcome of leukemia and small-cell lung cancer patients treated with etoposide (Ceppi et al., 2008; Kaufmann et al., 1994; Rossman et al., 2011; Uggla et al., 2007). Treatment sensitivity of glioma cell lines is further complicated due to genomic changes carried by cell models. Correlation between TopoII α expression and response to etoposide were evident but involvement of other molecular mechanisms in etoposide response is unavoidable. Ultimately, our study indicated that relationship between a single biomarker and targeted therapy is more complex than initially anticipated.

CHAPTER 4

NG2 EXPRESSION IN GLIOMA CELL LINES AND CONTRIBUTION TO DRUG RESISTANCE

4.1 INTRODUCTION

The role of NG2 in signalling pathways has been addressed in the literature extensively. NG2 by either its own signal transduction capacity or cooperating with $\beta 1$ integrins contributes to enhanced cell motility, survival and proliferation of the tumour cells and can act as a potent mediator of the resistance to therapies. The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin inhibitor, cilengitide, is currently under investigation in clinical trials for glioblastoma. However, the anti-tumorigenic effects of cilengitide and the factors that contribute to cilengitide resistance are not fully understood. In this study we investigated the inhibitory effects of cilengitide in glioma cell lines and the potential association between NG2 expression levels and response characteristics of the cells.

Working hypothesis of this chapter:

It has been hypothesised that glioma cell lines expressing high levels of NG2 exhibit significantly higher resistance to treatment with cilengitide than low-NG2-expressing cell lines. Cilengitide was selected due to its direct targeting of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins which allow us to investigate the contribution of NG2, predominantly co-localised with $\beta 1$ integrins, to cilengitide response.

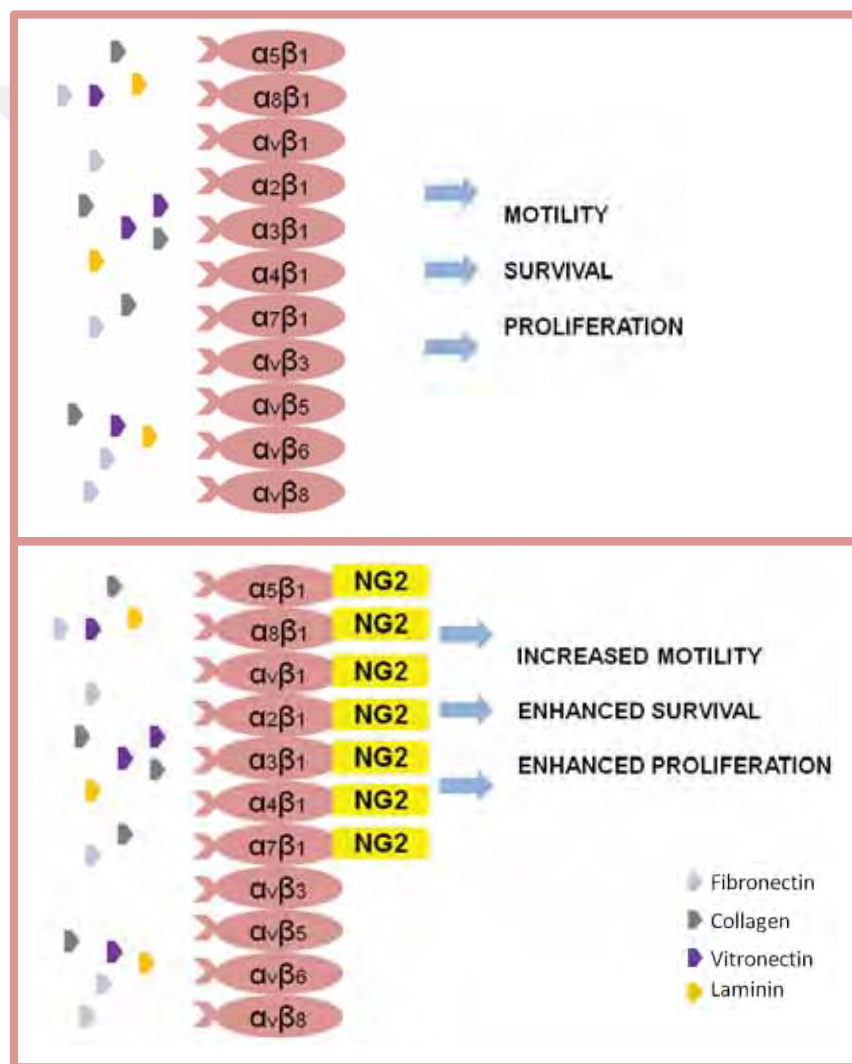


Figure 4.1 A schematic model illustrating our hypothesis that NG2 plays a role in drug resistance via stimulation of downstream cell proliferation, motility and survival associated pathways

The general aim of the study is to determine expression levels of NG2 in a panel of five glioma cell lines and to evaluate potential contribution of NG2 to drug resistance which will be assessed by monitoring the response of the cell lines to cilengitide.

More specifically, this study aims to:

1. Determine endogenous expression levels of NG2 at mRNA and total protein level in one osteosarcoma; MG-63 (used as a positive control) and the five glioma cell lines; T98G, A172, M059K, U-87 MG, M059J cell lines using immunoblotting and qRT-PCR.
2. Monitor chemotherapy response of the six cell lines to cilengitide to obtain IC₅₀ values.
3. Establish an association between expression level of NG2 in the cells and their cilengitide-response characteristics.
4. Examine whether the suppression of NG2 expression by siRNA knockdown alters response of A172 cells to cilengitide.

4.2 METHODS

4.2.1 Determining Efficiency of NG2 Gene Expression Using RT-qPCR

cDNA preparation from the RNA extracts of glioma cell are detailed in Section 2.4.3, Material and Methods, Chapter 2). RT-qPCR was performed on the cDNAs of T98G, A172, M059K, U-87 MG and M059J cells. The Taqman gene expression assay Hs00426981_m1 (CSPG4) (Applied Biosystems, Foster City, CA, USA) was used to amplify NG2 and RNA quantity was expressed relative to Eukaryotic 18S rRNA Endogenous Control (VIC/MGB Probe, Applied Biosystems, Mulgrave, VIC, Australia). The optimised efficiency of qRT-PCR reactions for NG2 and 18S were 89% and 100%, respectively.

4.2.2 Immunoblotting for NG2 protein

NG2 protein expression was detected using NG2 chondroitin sulfate proteoglycan mouse monoclonal antibody (Millipore Corporation, Billerica, MA, USA). The protocols described in Section 2.5.3, Material and Methods, Chapter 2 provide more detailed information about the steps of immunoblotting for NG2 protein.

4.2.3 Preparation of Cilengitide

Cilengitide (EMD 121974) (Merck) was supplied in physiological saline solution at 25 mM (15 mg/ml) concentration and stored at 4 °C. Cilengitide concentrations ranging from 0.25 to 500 µM were tested to determine IC₅₀ of cilengitide for each cell line.

4.2.4 Transfection of Cells Using Nucleofection Technique

A172 cells were grown in T75 culture flasks until the monolayer reached 80% confluency. Cells were trypsinised and centrifuged as described in Section 2.2.1, Material and Methods, Chapter 2. The cell pellet was resuspended and manual cell counting was performed using the haemocytometer as detailed in Section 2.2.6,

Material and Methods, Chapter 2. After counting, required number of cells dispensed into 15ml centrifuge tubes and centrifuged at 200 x g for 10 minutes. The pellet was resuspended in room temperature nucleofection solution (100 µl) (Cell line Nucleofector ® Kit T, Lonza Australia, Pty. Ltd, VIC, Australia) and 2.5 µg Silencer Select Pre-designed siRNA (CSPG4; s3651, s3652, s3653, Reference Sequence NM_001897.4, Applied Biosystems/Ambion, Austin, TX, USA) or 2.5 µg Allstars™ Negative Control siRNA (Qiagen, Hilden, Germany) was added. The sample was transferred into an Amaxa specified transfection cuvette (Cell line Nucleofector ® Kit T, Lonza Australia, Pty. Ltd, VIC, Australia) and the transfection was performed on a Nucleofector™ II device (Lonza, Walkersville, MD, USA). Pre-warmed culture medium was then added to the cells in the cuvette and cells were transferred into plates which were filled with pre-incubated medium. Final number of cells per well of culture plates are shown in Table 4.1. Re-plated cells were allowed to incubate in a humidified atmosphere with 5% at 37 °C for 24 hr before addition of cilengitide. Following 24 hr incubation, medium was changed with fresh medium containing different dilutions of cilengitide (1 to 15 µM). Medium in wells allocated for untreated control group was refreshed with culture medium, culture plates were then incubated for another 24 hr. RNA and protein extractions were conducted as described in Section 2.4.1 and 2.5.1 of Material and Methods Chapter, respectively.

Table 4.1 Number of cells plated for control and transfected groups

Culture Plate	Number of Cells / well (untransfected control)	Number of Cells / well (transfected groups)
96-well plate	7x10 ³	1x10 ⁴
12-well plate	2.5x10 ⁵	3.25x10 ⁵
6-well plate	5x10 ⁵	6.5x10 ⁵

4.2.5. TUNEL Assay

The TUNEL assay measures nuclear DNA fragmentation which is a feature of apoptotic cells. *In situ* cell detection kit, POD (Roche Diagnostics, QC, Canada) was used to detect DNA damage by labelling free'-OH termini in the DNA breaks. TUNEL assay was performed to investigate cilengitide-induced apoptosis in CSPG4 (NG2) siRNA-52 transfected and untransfected A172 glioma cell line. Since cilengitide caused detachment of cells in culture, it was impractical to grow cells on slides after cilengitide treatment. Therefore, a cytopspin method was used to spin a cell suspension containing 1×10^5 cells in a final volume of 200 μ l and produce a monolayer of cells on slides (StarFrost® Microscope Slides, Poly-L-Lysine coated, Proscitech, Thuringowa, QLD, Australia). Cell suspensions were cyto-centrifuged at 800 rpm for 3 minutes using a Shandon Cytospin II (Shandon Instruments, Astmoor, UK). A hydrophobic line was applied around the cyto-centrifuged cells using a pen (Dako pen, Dako, Glostrup, Denmark) and cells were immediately fixed with 4% paraformaldehyde (PFA) solution for 1 hour in a humidified chamber. The slides were rinsed three times with PBS and treated with blocking buffer (3% H₂O₂ in methanol) for 10 minutes at 4 °C. Then, slides were rinsed with PBS and incubated with permeabilisation solution for 2 minutes on ice. Following permeabilisation, one untransfected and untreated sample allocated for positive control was treated with DNase Buffer (Recover All Total Nucleic Acid Isolation Kit, Applied Biosystems/Ambion, Austin, TX, USA) to cause DNA fragmentation, prior to labelling procedures. All samples were washed again three times with PBS, the residual liquid removed before applying the TUNEL reaction mixture as per protocol. Negative control was treated with Label Solution only without Enzyme Solution. Slides were incubated for 60 minutes in a humidified chamber at 37 °C and followed by three rinses with PBS. Any residual liquid on samples was removed. Signal

conversion was conducted by the addition of anti-fluorescein antibody POD conjugate (50µl). The samples were then incubated for 30 minutes in a humidified chamber at 37°C and washed again three times with PBS. TUNEL-positive (brown) colour development was performed by incubating all slides with Substrate, Liquid DAB+ Chromogen Kit (Dako, Glostrup, Denmark) for 5 minutes. All samples were stained with haematoxylin, Harris Solution (Fronine [Thermo Fisher], NSW, Australia) which allowed the nuclei to be coloured blue and visible for TUNEL-negative cells. Slides were air-dried for 10 minutes in a fume hood and mounted with Ultramount (Fronine [Thermo Fisher], NSW, Australia) using coverslips. The slides were examined under a light microscope. For TUNEL-positive cells, a brown staining within the nucleus or cytoplasm of the cells was observed. The cells stained intensely dark brown are regarded as TUNEL-positive and counted for analysis.

4.3 RESULTS

4.3.1 Determining Endogenous Expression Levels of NG2 in One Osteosarcoma and the Five Glioma Cell Lines and Their Responses to Cilengitide.

The expression of NG2 at both total protein and mRNA levels were determined using immunoblotting and qRT-PCR techniques, respectively, in T98G, A172, M059K, M059J, U-87 MG and MG-63 (positive control) cells. NG2 protein was detected in A172, U-87 MG at levels comparable to NBORF. Negligible levels of NG2 were detected in M059K, M059J and T98G cells (see Figure 4.2, A and B). The same trends were not observed at the mRNA level with highly variable expression detected. Consistent with A172 protein expression, NG2 mRNA was also higher than that in normal brain. However Significantly low NG2 mRNA expression was measured in T98G cells. (see Figure 4.2, C).

The proliferative response of the each cell line to cilengitide treatment was screened to determine IC_{50} doses of the drug at 24 h after treatment using MTS cell proliferation assay (see Figure 4.3). Among the all cell lines, viability of A172 and MG-63 cells were effectively inhibited by 50% at 7 and 1.5 μ M cilengitide concentrations, respectively. In contrast, U-87 MG cells were found to be relatively resistant to cilengitide treatment with an IC_{50} of 100 μ M. However, other cell lines; T98G, M059K and M059J were highly resistant to cilengitide, with IC_{50} values up to 500 μ M. Unexpectedly, A172 expressing high levels of NG2 and U-87 MG with moderate NG2 expression represented the extremes of sensitivity to cilengitide. Moreover, NG2 positive MG-63 cells found to be the most sensitive to cilengitide treatment.

M059K and T98G exhibited the most similar pattern of changes in the IC_{50} to that found for U-87 MG. A 47% decrease in cell viability was observed for T98G and

M059K at 100 μ M, however cell viability increased to 55-60% at more concentrated doses of the drug (250 and 500 μ M). M059J cells experienced a 50% loss of viability at 50 μ M of cilengitide, however, increasing concentrations of cilengitide (250 and 500 μ M) resulted in an increase in the number of viable cells. Therefore, T98G, M059K and M059J cell lines had a similar response pattern. In contrast, although IC_{50} obtained for U-87 MG cells was not found to be statistically significantly different than IC_{50} values obtained for T98G, M059K and M059 cell lines, U-87 MG cells exhibited a steady decrease after reaching IC_{50} .



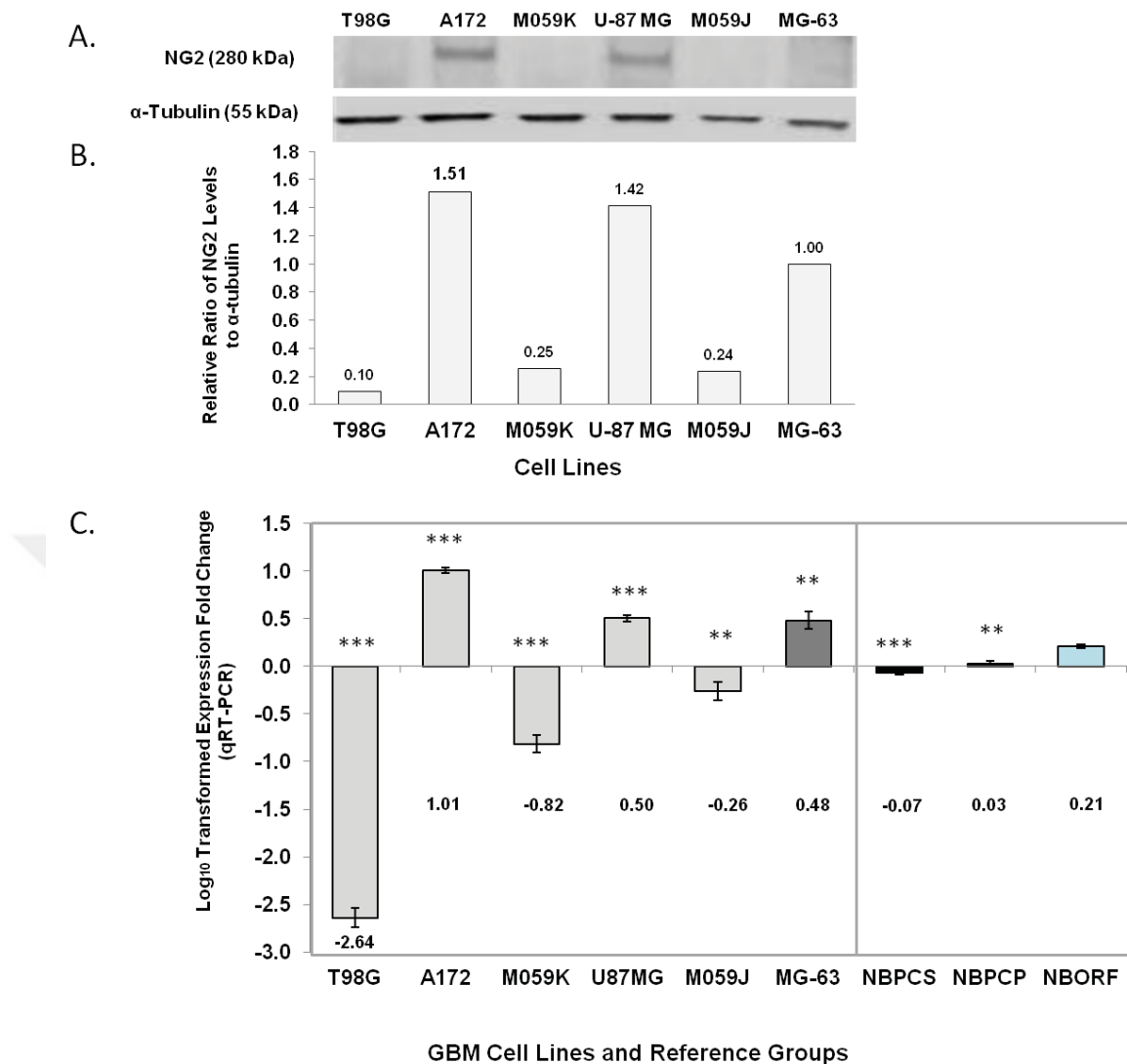


Figure 4.2 NG2 expression at total protein and mRNA level in the five glioma and one osteosarcoma cell line.

(A and B) Immunoblotting of the whole cell lysates. NG2 expression levels were detected using a rabbit polyclonal antibody against NG2. (C) qRT-PCR analysis of NG2 normalised to 18S levels. The expression fold change was calculated relative to human brain reference sample, NBTot. Statistical significance of differences was calculated relative to NBORF.

Data are representative of three experiments. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

Abbreviations: ns: non-significant, NBPCS: Human Parietal Cortex Superior Brain Total RNA, NBPCP: Human Parietal Cortex Posterior Brain Total RNA, NBORF: Human Orbital Frontal Cortex Brain Total RNA

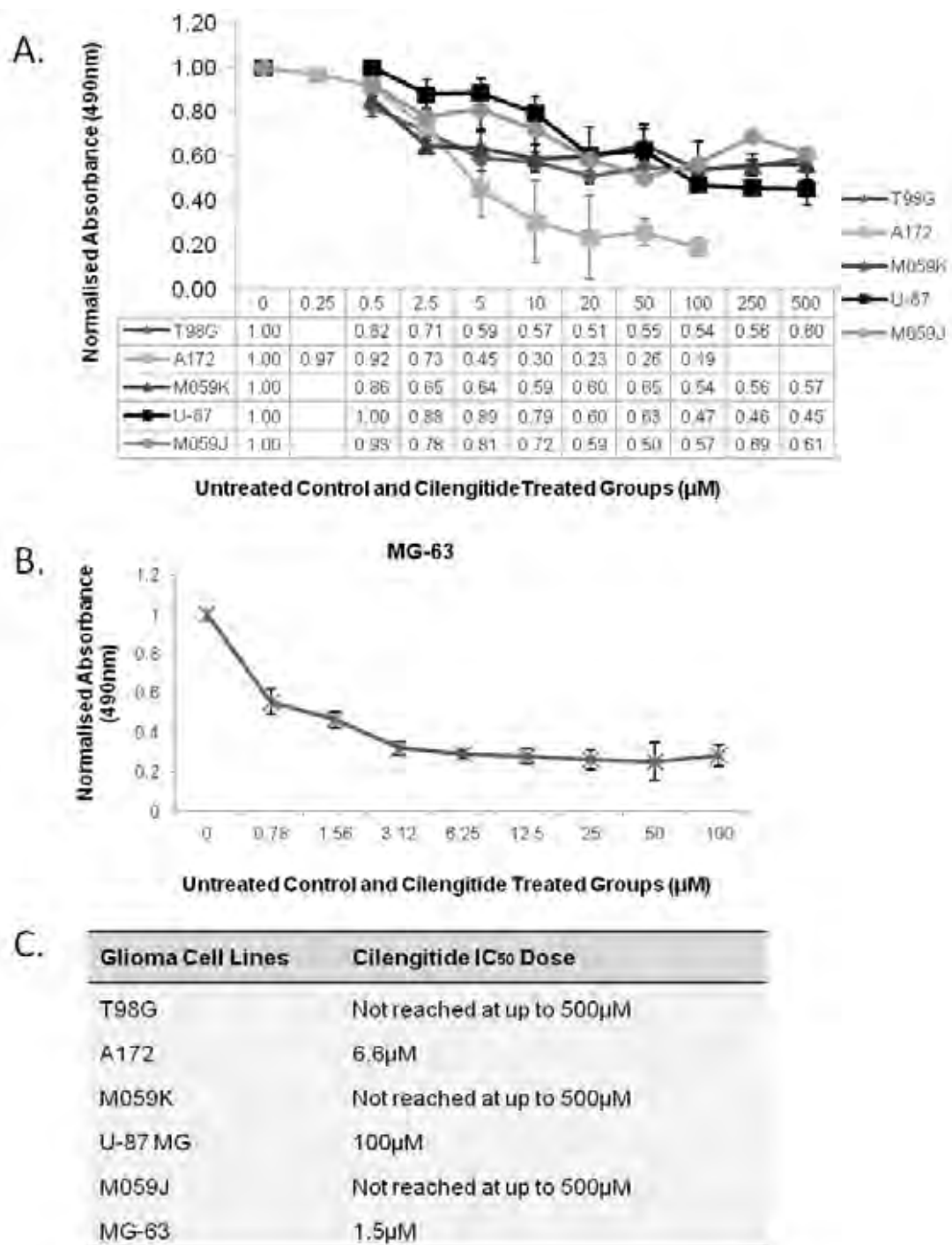


Figure 4.3 Inhibitory effects of cilengitide on glioma cell lines and a NG2-positive control cell line (24 hr treatment)

(A) Dose-response curves for glioma cell lines and (B) positive control. (C) The cell viability of the cell lines was determined by MTS cell proliferation assay and IC₅₀ values were calculated. Each point represents the mean $\pm$ S.E. of three independent measurements.

4.3.2 Knockdown of NG2 Expression in A172 Cells

Other factors are involved in resistance to cilengitide. To isolate the role NG2 plays in mediating resistance, we designed experiments to assess whether the knockdown of NG2 interferes with the sensitivity of glioma cells to cilengitide. Transient knockdown of NG2 was performed using NG2 small interfering RNA (siRNA) in the A172 cells which are high expressors of NG2 (shown in Figure 4.4, A and B). Because the siRNA against NG2 was not validated, 3 potential siRNAs were tested.

Among the all siRNAs (siRNA-51, siRNA-52, siRNA-53) examined, siRNA-52 suppressed the NG2 expression at a high level. NG2 protein expression was inhibited by 50% and NG2 mRNA levels suppressed by 84% at 24 hr following transfection. No significant effects upon protein and mRNA were observed when transfecting cells with the Allstars negative control and transfection media only control. siRNA transfection was detected to be more efficient at mRNA level than total NG2 protein at 24 hr treatment (see Figure 4.4, C).

Inhibition of NG2 with siRNA-52 was assessed at 24, 48 and 72 hr post-transfection. While there was no substantial decrease of NG2 expression at total protein level at 24 hr after transfection, the expression levels were inhibited by 65% at 48 hr and 62% at 72 hr compared with the controls. (see Figure 4.5, A and B). At the mRNA level, NG2 expression was markedly low, with 89, 60 and 56% inhibition compared to controls at 24, 48 and 72 hr post-transfection, respectively (see Figure 4.5, C). As this experiment was being conducted, a rather odd phenomenon was observed. Basal expression levels of NG2 were unstable in the control cells.

Several parameters such as FBS concentration in medium supplemented with 1 and 10% (standard culture medium), refreshing medium every 24 hr and viability of cells

following transfection were evaluated to stabilise both endogenous NG2 expression levels and to optimise siRNA transfection conditions. The use of classical culture medium supplemented with 10% FBS demonstrated higher NG2 mRNA expression levels in untransfected A172 cells compared to the cells maintained in medium containing 1% FBS. (see Figure 4.6, A). Refreshing culture medium (10% FBS) every 24 hours showed slightly improved mRNA expression levels at each time point, shown in Figure 4.6, B. Viability of the cells following transfection was also examined. A dramatic decrease in cell viability was observed when cells were transfected with the nucleofector reagent. In transfected groups, an average of ~30% mortality rate was detected (see Figure 4.6, C; light-gray bars). To counteract this loss of viability, the cell number per well was increased by ~40% from 7×10^3 to 1×10^4 cells/mL. T Figure 4.6, C (dark-gray bars) demonstrates a greater cell viability rate in each transfection group.

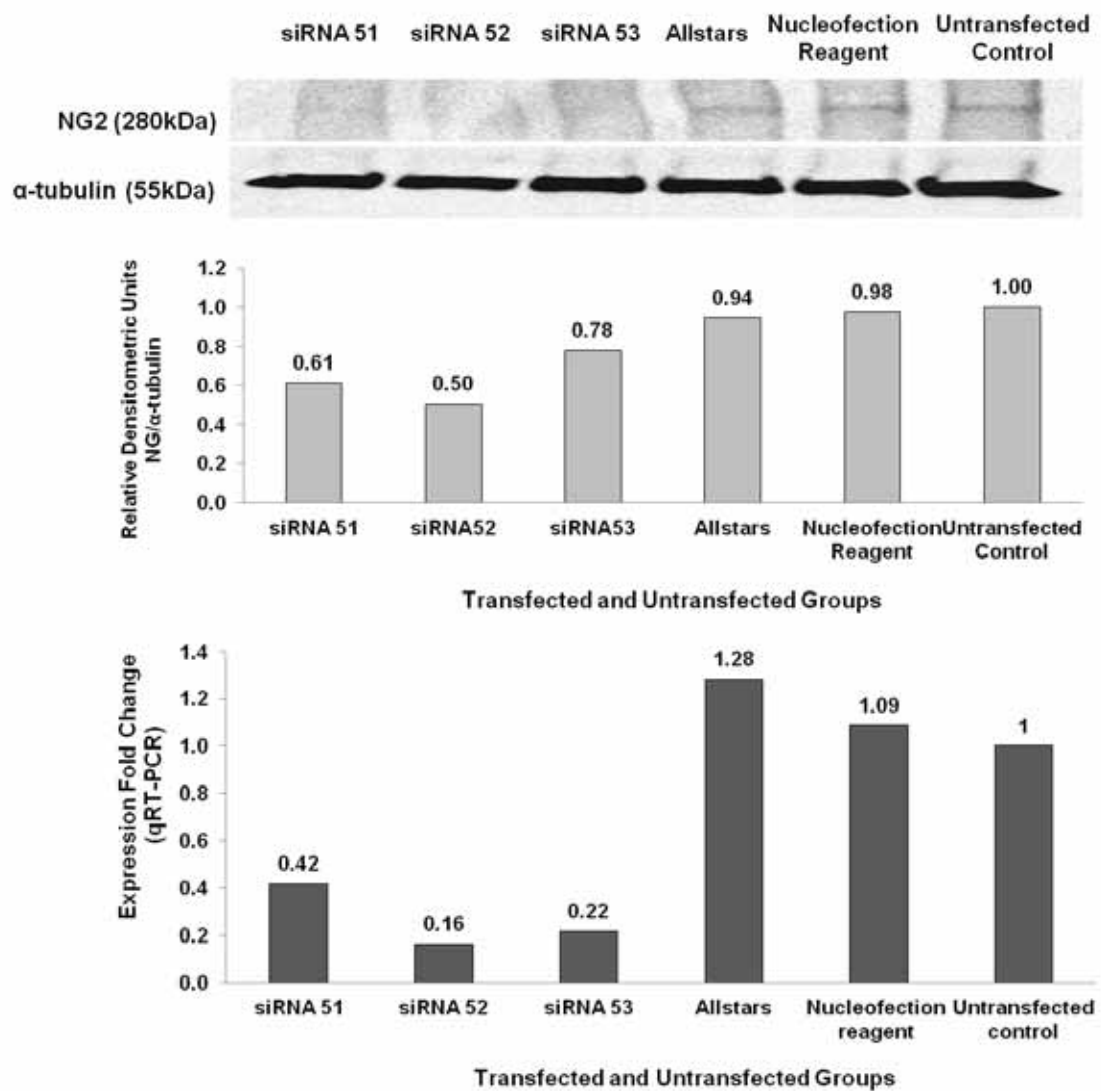


Figure 4.4 Knockdown of NG2 in the A172 cell line (24 hr)

A172 cells were transfected via electroporation (Amaxa) with siRNA-51, siRNA-52, siRNA-53. As controls, cells were transfected with Allstars negative control and nucleofector reagent only (no siRNA). **(A and B)** NG2 protein expression in treated and untreated cells at 24 hr was detected using a rabbit polyclonal antibody against NG2 by immunoblotting. **(C)** qRT-PCR was performed to examine mRNA expression levels of NG2.

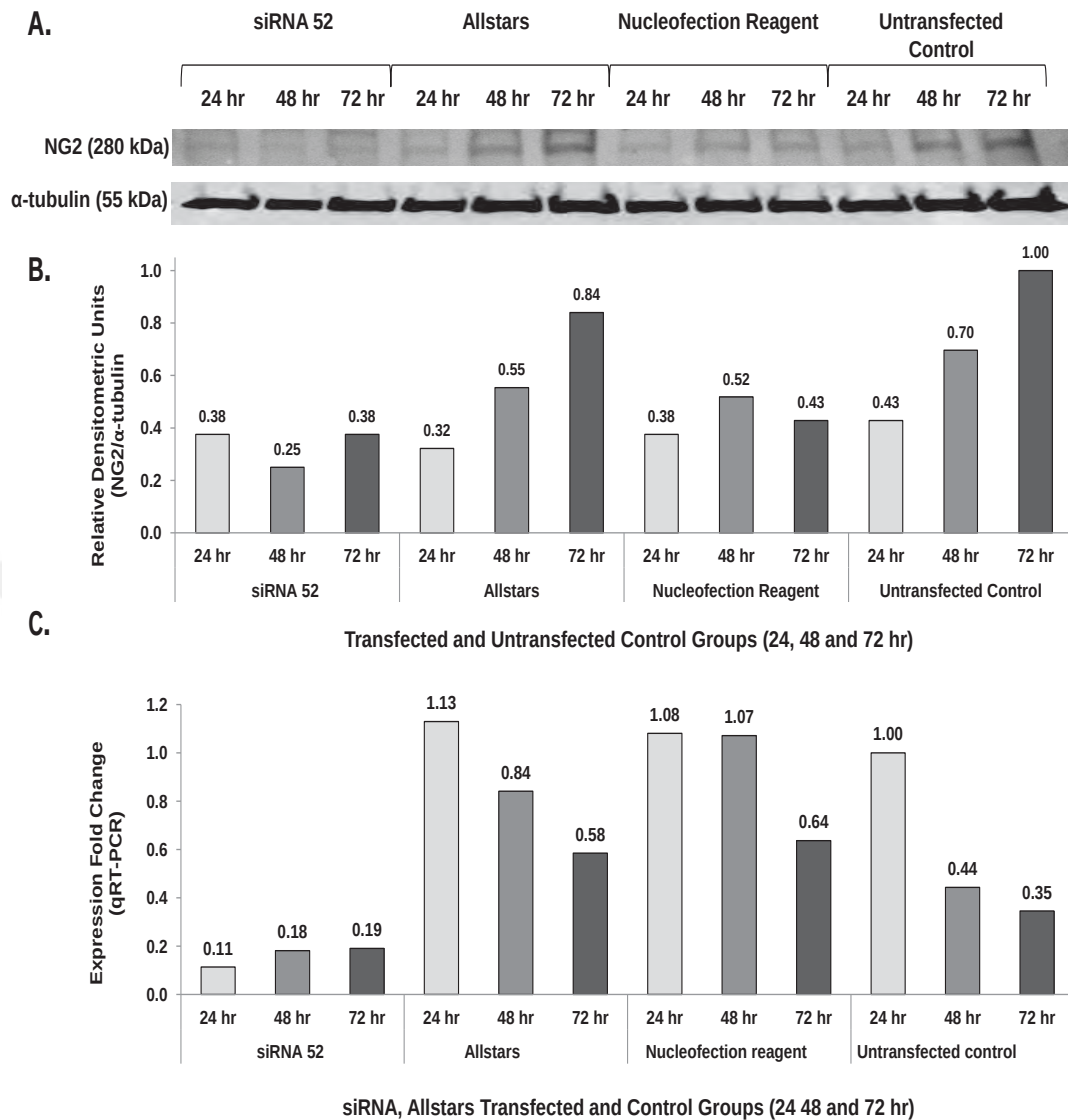


Figure 4.5 Knockdown of NG2 in A172 cells at 24, 48 and 72 hr after siRNA-52 treatment

A172 cells were transfected with either siRNA-52, Allstars negative control and nucleofector reagent only. The experiment was monitored for 72 and the cells were collected for RNA and total protein extractions at three time points, 24, 48 and 72 hr after transfection. **(A and B)** NG2 protein expression was detected using a rabbit polyclonal antibody against NG2 by immunoblotting. **(C)** qRT-PCR was performed to examine mRNA expression levels of NG2.

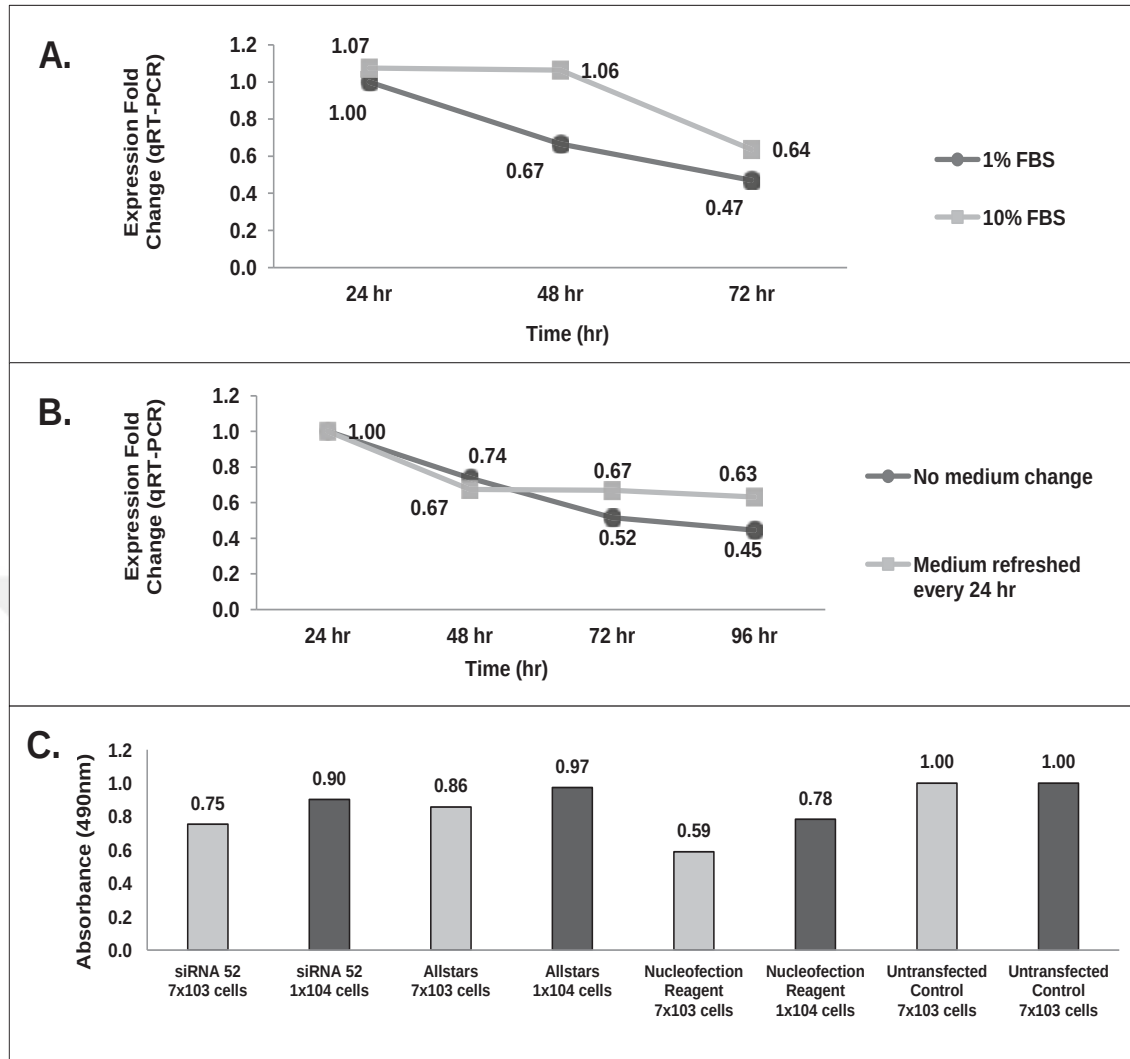


Figure 4.6 Parameters examined to improve endogenous expression levels of NG2 and transfection conditions

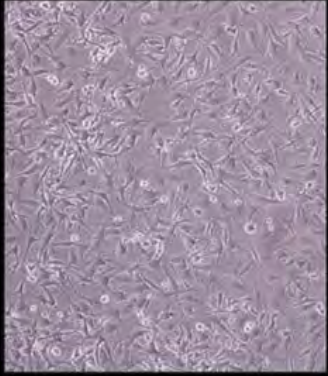
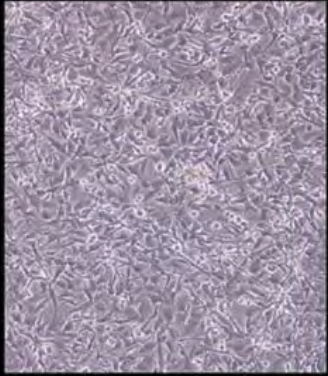
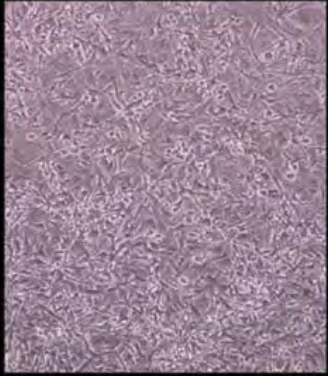
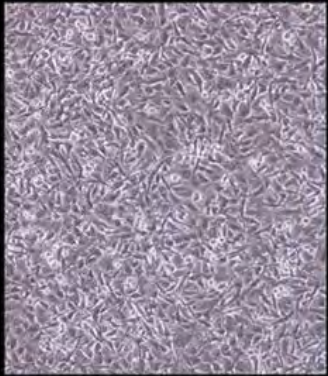
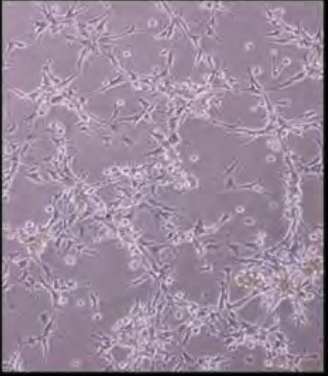
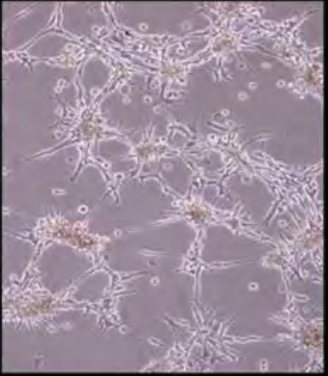
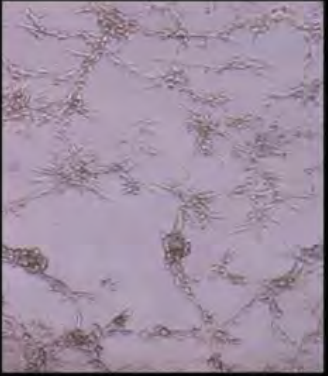
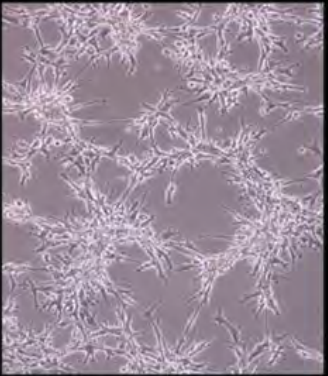
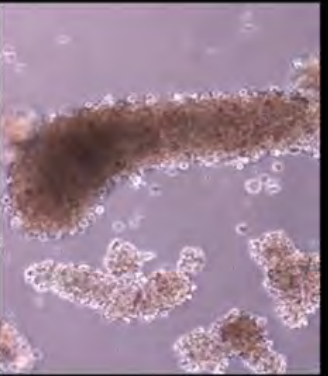
(A) Medium supplemented with 10% (standard culture medium) and 1% FBS was tested to determine if NG2 expression levels could be stabilised. **(B)** The effect of culture medium-refreshing (10% FBS) every 24 hour was also assessed. **(C)** Mortality rate (~30%) following transfection using electrofection method was determined. Survival rates of transfected cell groups were examined with increased cell number per well of 96-well plate compared to the viability of the untransfected cells.

4.3.3 Monitoring the Cilengitide-response of the Transfected and Untransfected A172 Cells

The morphology of cells treated with siRNA-52, Allstars negative control and nucleofector reagent transfected and untransfected A172 cells at 24 hr post-treatment with cilengitide was examined (see Figure 4.7, A). A172 cells treated with 3 μ M cilengitide induced the detachment of the cells from the monolayer within 24 hours. Increasing the cilengitide dose from 3 to 7 μ M resulted in an enhanced detachment of the cells in a concentration-dependent manner. Untransfected and all transfected cell groups formed clumps in response to increased concentration of cilengitide (7 μ M).

Cell proliferation, measured with the MTS assay showed gradually increased cytotoxicity in response to increased cilengitide concentrations. Viability of the siRNA transfected cells was found to be significantly lower compared to the observed cell viability of the untransfected, and other negative control groups (see Figure 4.7, B).

Viability of these cell clumps was also assessed with the vital stain, trypan blue. The cell clumps were gently passed through a sterile pipette tip until the cell clumps disappeared. Cell counting by trypan blue staining showed that in siRNA-52 transfected cells, cilengitide treatment with 7 μ M resulted in reduced number of live cells (66%) at 24 hr post-treatment. Knocking down of NG2 was not seem to restore resistance to cilengitide. Although not statistically significant, level of cell mortality observed in siRNA-52 transfected cell group (34%) was found to be increased compared to the untransfected and transfection control groups (18%) treated with cilengitide (7 μ M) (see Figure 4.7, C).

A.	siRNA 52	Allstars	Nucleofection Reagent	Untransfected Control
Untreated				
3µM Cilengitide				
7µM Cilengitide				

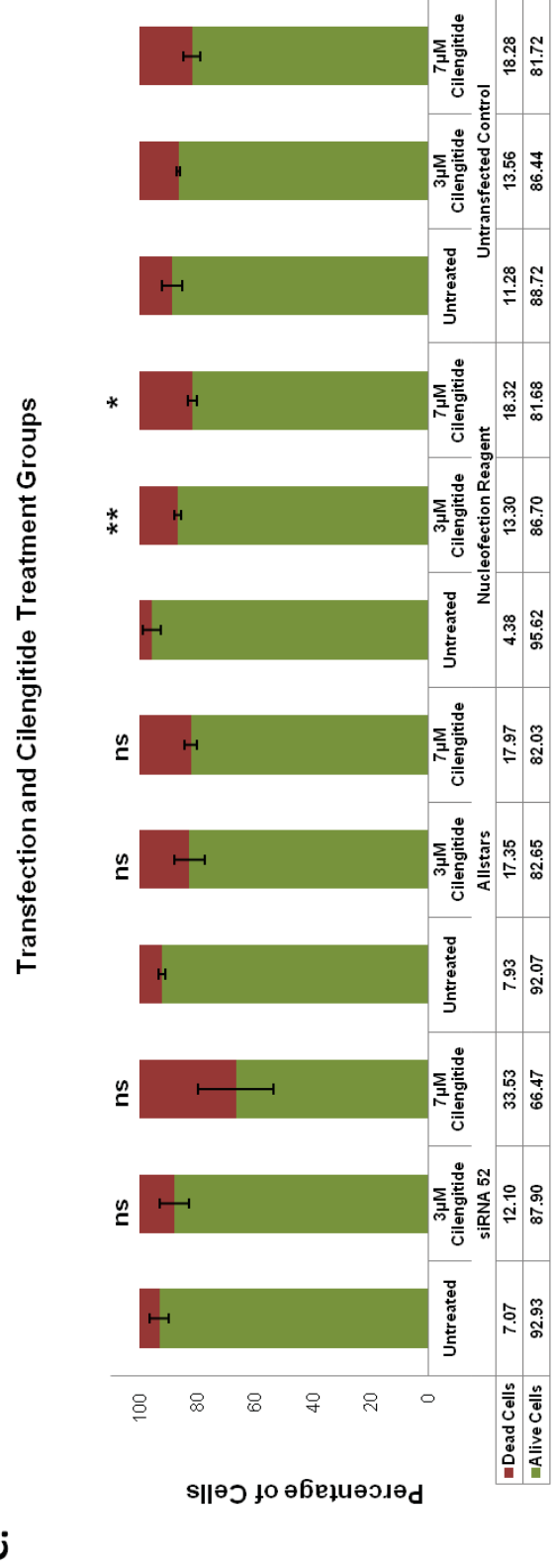
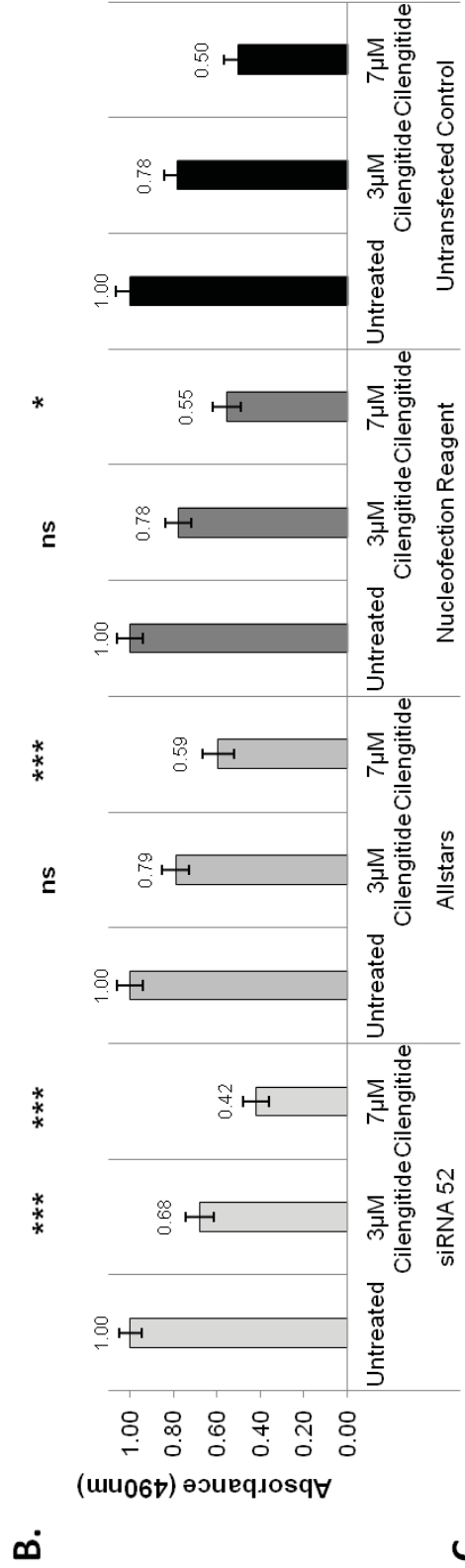


Figure 4.7 Cilengitide responses of the A172 cell groups, transfected vs untransfected and cilengitide-treated vs –untreated, detected by both trypan blue counting and MTS cell proliferation assay

Cilengitide was added at 24 hours post-transfection and, MTS assay and cell counting were performed at 24 hr following addition of the drug. The cells were collected at 24 hr after addition of cilengitide and therefore at 48 hr following transfection.

(A) Representative light microscopy images of the A172 cells transfected with siRNA-52, Allstars negative control and nucleofector reagent and treated with 3 and 7 μ M (IC₅₀ dose of cilengitide for A172) of cilengitide. MTS assay was performed to detect viable cells in culture. **(B)** The figure shows the percentage of viable cells in culture. **(C)** Viability of the cells was also assessed by trypan blue counting method. Results are expressed as percentages of dead and viable cells.

Values are mean of the measurements obtained from 12 wells which were designated as cilengitide treated or untreated transfected and untransfected test groups.

Statistical significance of differences was calculated relative to untransfected treatment groups. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

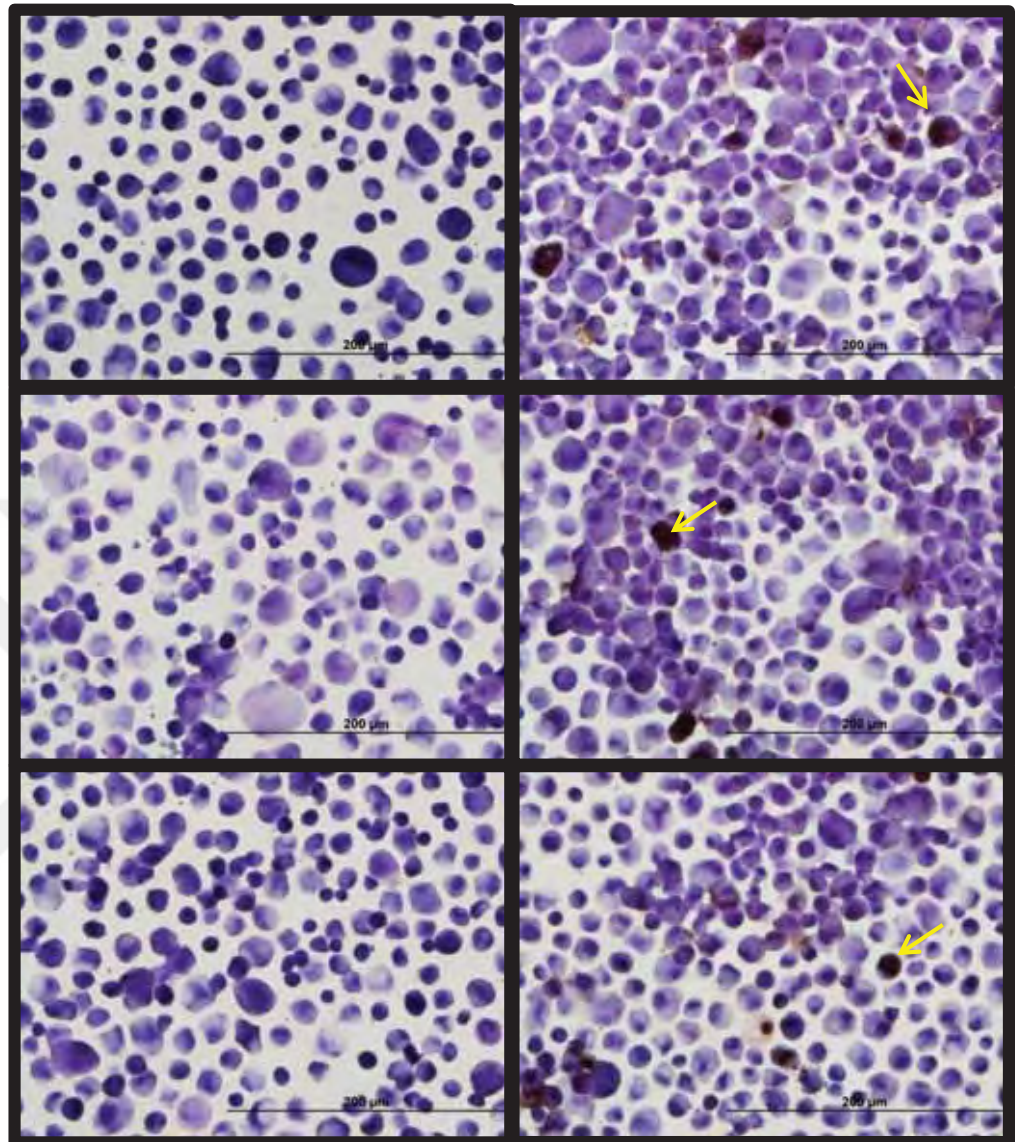
4.3.4 Cilengitide-induced Apoptosis in siRNA-52-transfected and -untransfected A172 Cells

The degree of apoptosis in siRNA-52-transfected and untransfected cells was assessed using TUNEL assay after cells were treated with cilengitide. No TUNEL positive nuclei were detected in negative control cells. Isolated TUNEL-positive cells observed as intense dark brown staining in positive control were indicative of apoptotic cells. TUNEL staining of untransfected cells demonstrated a dramatic increase (10-fold, $p < 0.01$) in apoptotic cells following cilengitide treatment (7 μM) compared to untreated-untransfected cell group. Cilengitide treatment of nucleofection reagent transfected cells also resulted in an increase in apoptotic cells by 4-fold ($p < 0.001$) compared to its untreated control group. The number of apoptotic cells detected in siRNA-52 transfected-treated vs. siRNA-52 transfected-untreated cell group was found to be slightly increased following treatment. However, there was not a statistically significant difference between treated and untreated siRNA52 transfected groups. Unexpectedly, cilengitide treatment at 7 μM did not show a considerable level of induction in number of apoptotic cells (see Figure 4.8). Although it is variable, a mortality rate of up to 18% was also noted in transfected (siRNA-52, allstars negative control and nucleofector reagent) but not in untreated cells.

A.

Negative Control

Positive Control

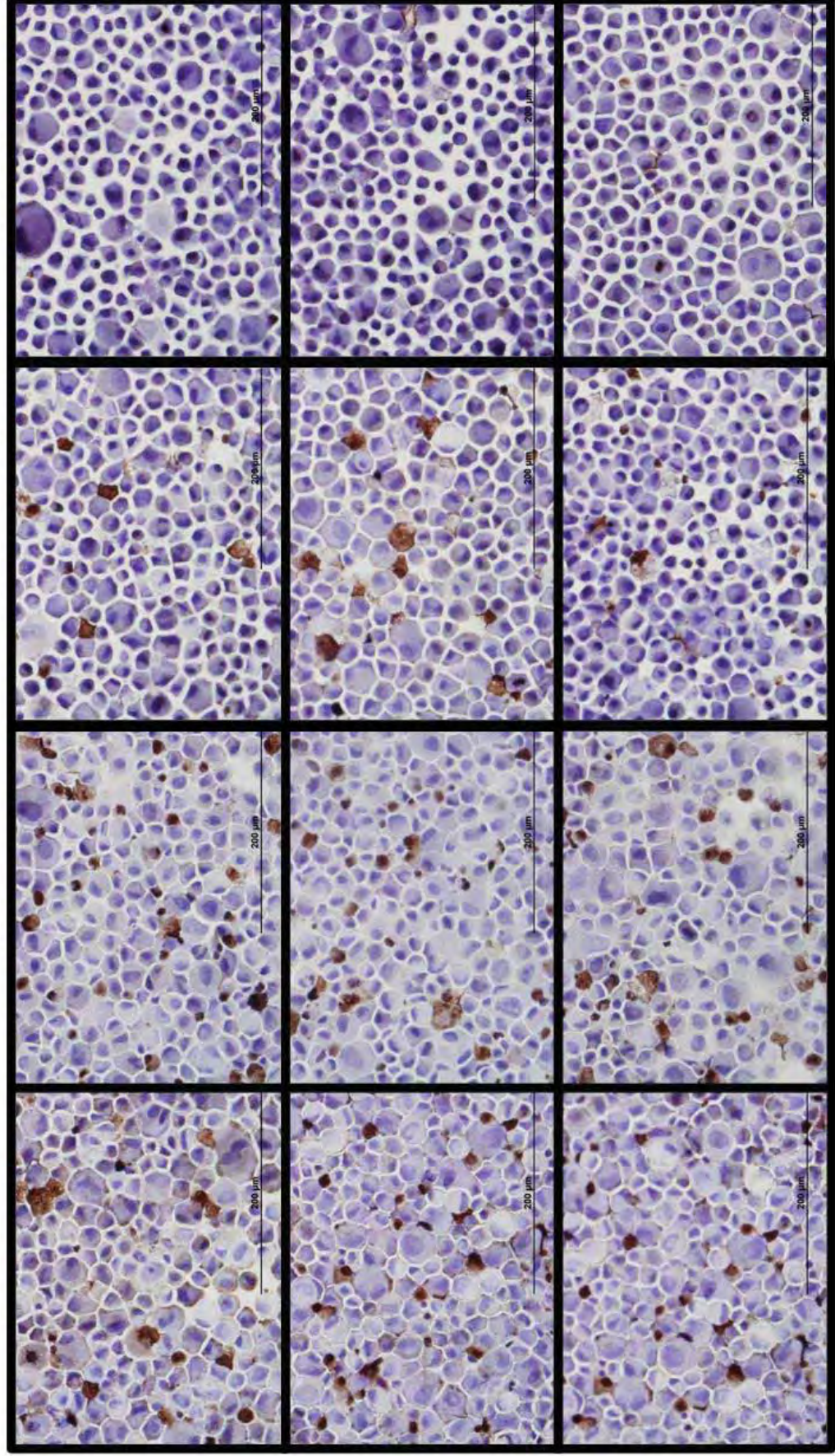


B. siRNA 52 Transfected & Untreated

Allstars Transfected & Untreated

Nucleofection Reagent Transfected & Untreated

Untransfected & Untreated

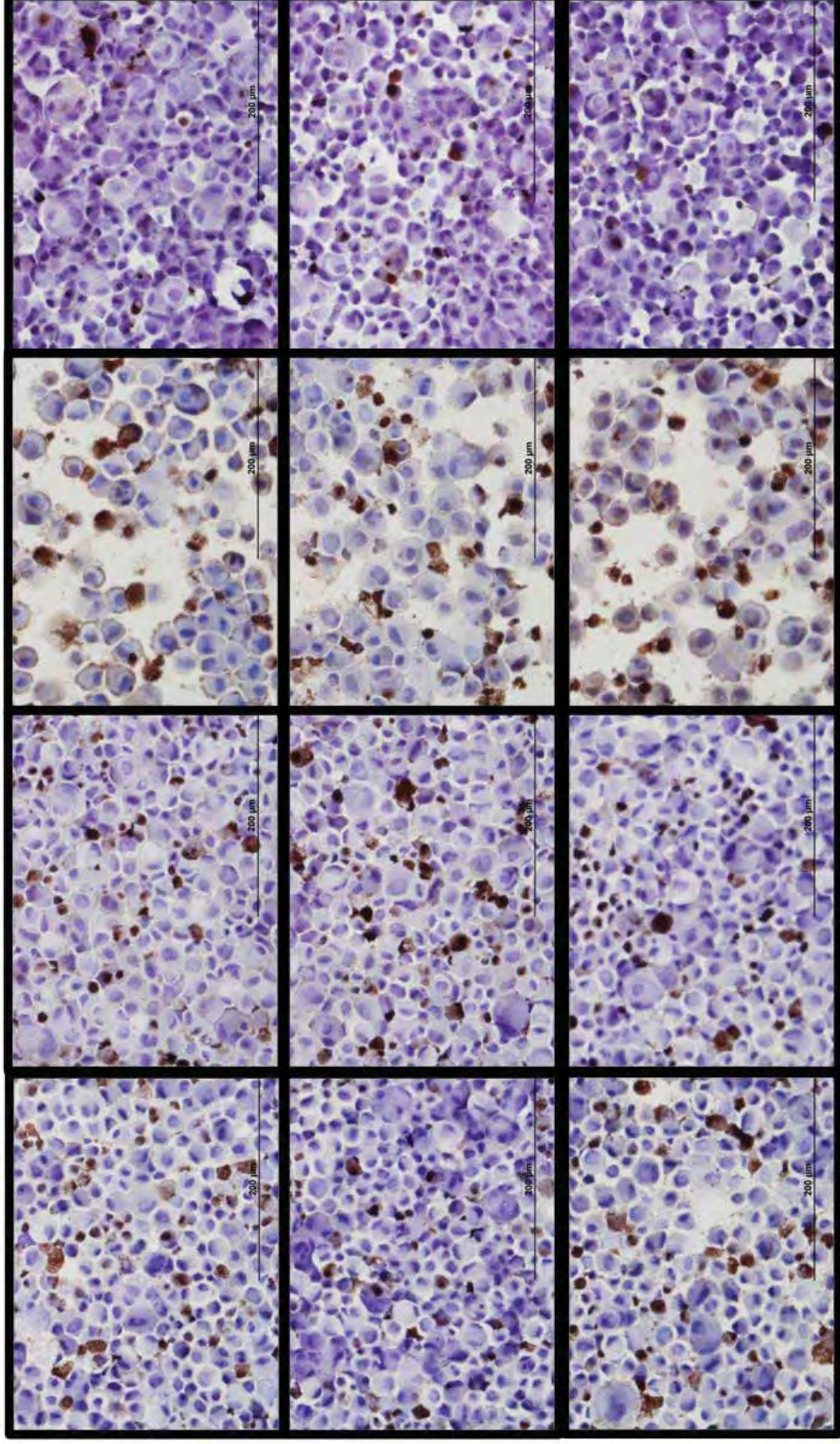


C. siRNA 52 Transfected & Treated 7 μ M Cilengitide

Allstars Transfected & Treated 7 μ M Cilengitide

Nucleofection Reagent Transfected & Treated with 7 μ M Cilengitide

Untransfected & Treated with 7 μ M Cilengitide



D.

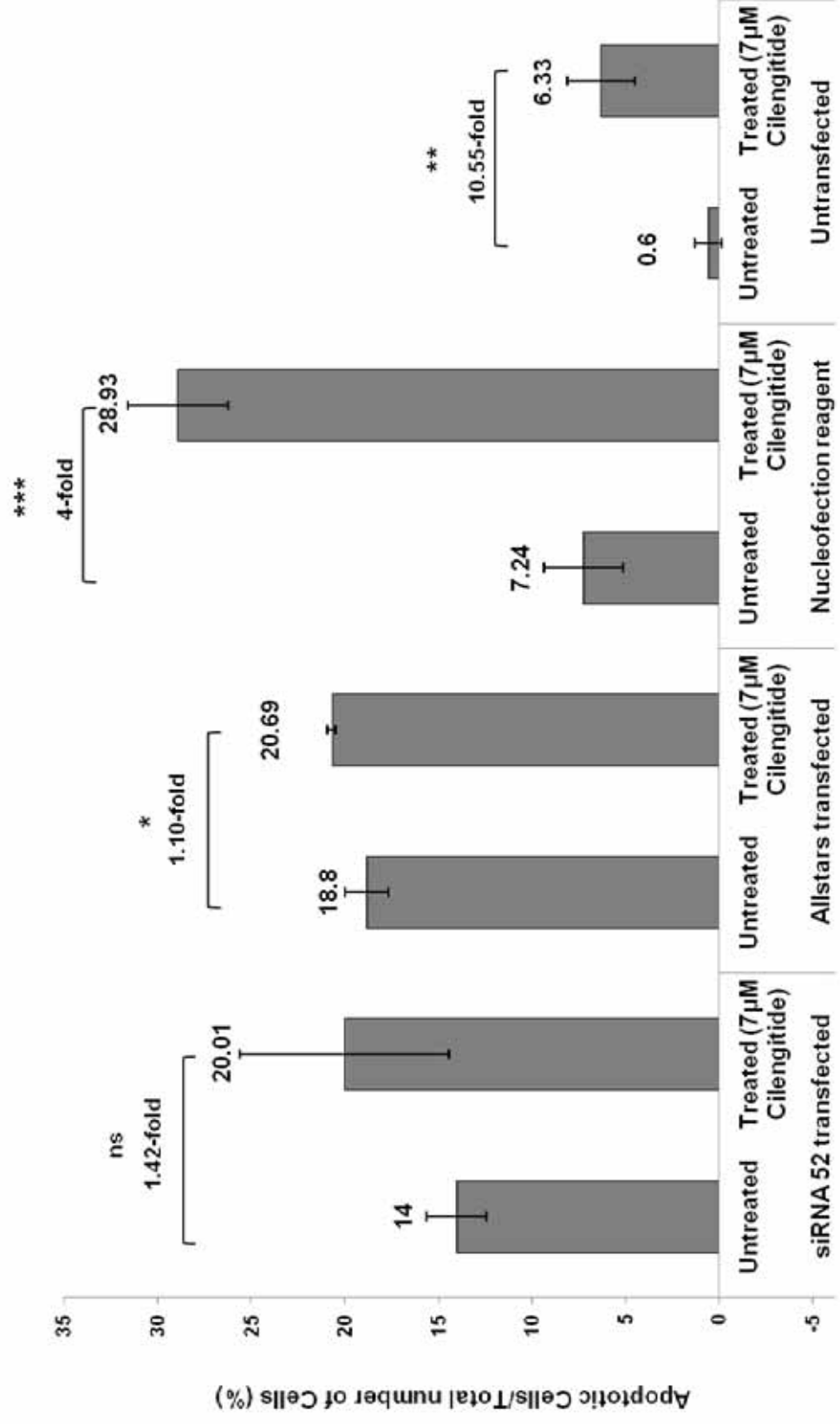


Figure 4.8 TUNEL staining of NG2 knock down cells treated with cilengitide

Cells which stained brown by the TUNEL assay were considered apoptotic, while blue-stained cells (stained with hematoxylin) were regarded as non-apoptotic.

(A) Isolated TUNEL-positive cells were seen in the positive control (DNase induced apoptosis). The yellow arrows points to TUNEL positive cells. (B) Representative images of untransfected and siRNA-52, Allstars negative control, nucleofection reagent transfected and untreated control groups, 40X magnification. (C) Representative images of cilengitide treated untransfected and transfected A172 cells, 40X magnification. (D) The graph shows the percentage of apoptotic cells at 24 hr after treatment with 7 μ M cilengitide.

Statistical significance of differences was calculated relative to untreated cells in each group. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

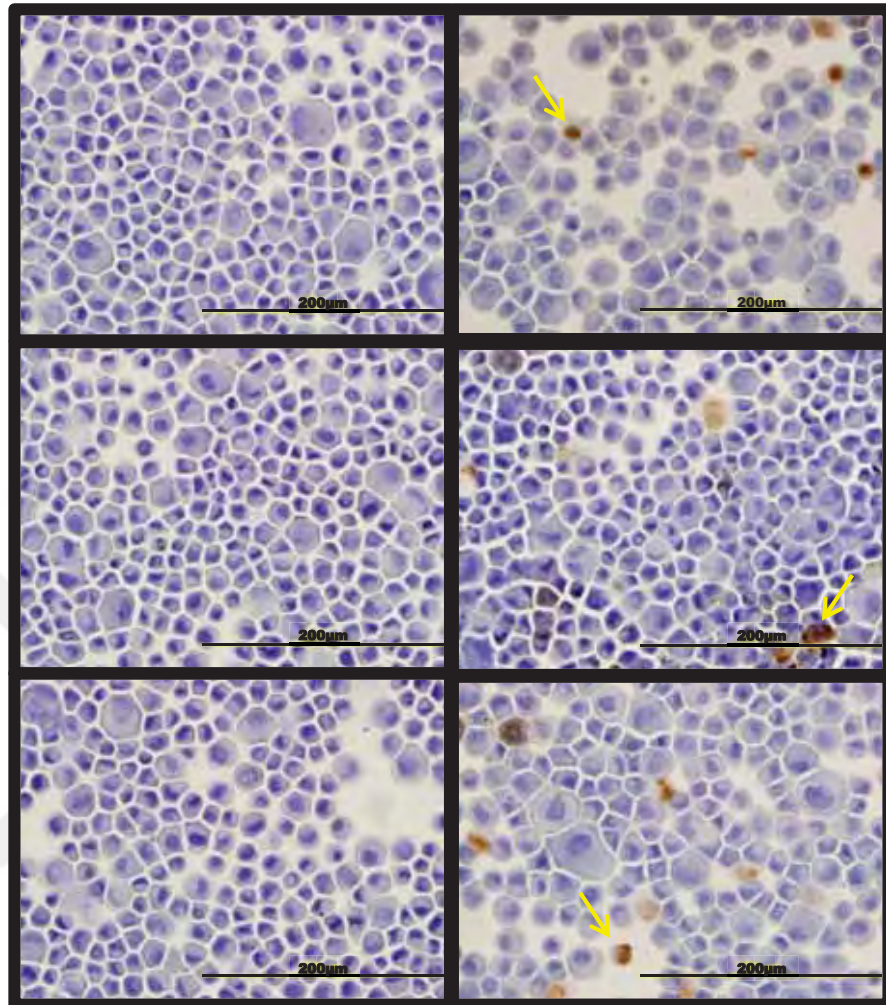
4.3.5 Cilengitide (7, 10, 15 μ M)-induced Apoptosis in Untransfected and siRNA-52-Transfected A172 cells

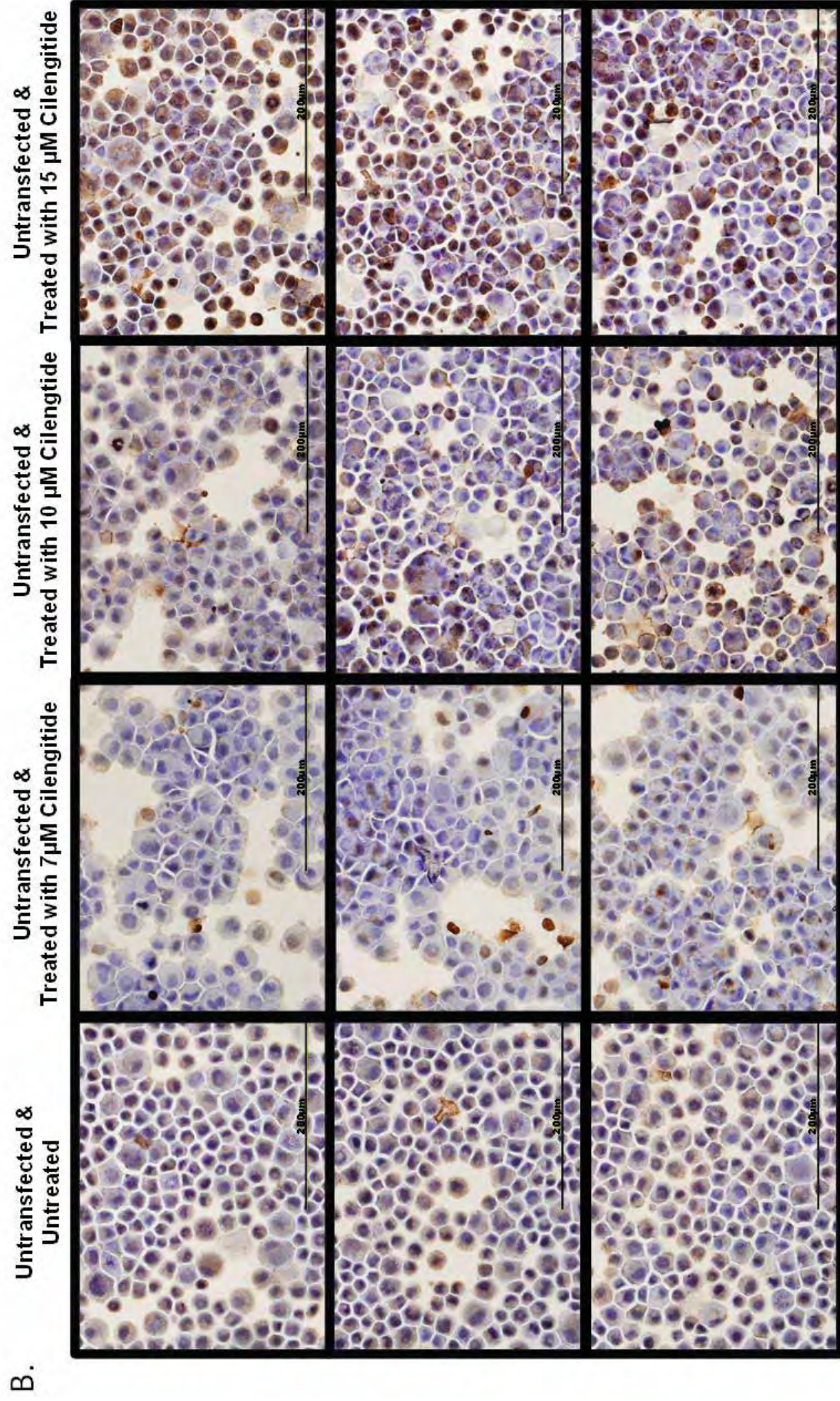
DNA fragmentation induced by three different cilengitide doses, 7, 10, 15 μ M, was measured in untransfected and siRNA-52 transfected cells (see Figure 4.9). Lower mortality rates in siRNA-52 transfected group were also recorded. The rate of TUNEL-positive apoptotic cells was increased in a cilengitide dose-dependent manner in both untransfected and siRNA-52 transfected groups. The number of apoptotic cells at each drug concentration was compared to untreated control groups of the transfected and untransfected groups. Cilengitide (15 μ M) induced apoptosis in 14% of the cells in untransfected control group compared to 20% of apoptotic cells present in the siRNA transfected group at the same drug concentration. Although the rate of apoptotic cells was detected to be slightly higher in untransfected cells (7.31-fold) treated with 15 μ M cilengitide compared to siRNA-52 transfected cells (6.67-fold) exposed to the same treatment dose, the difference was not statistically significant. This was assessed by comparing the number of positive nuclei observed in untreated control cells of each transfection group.

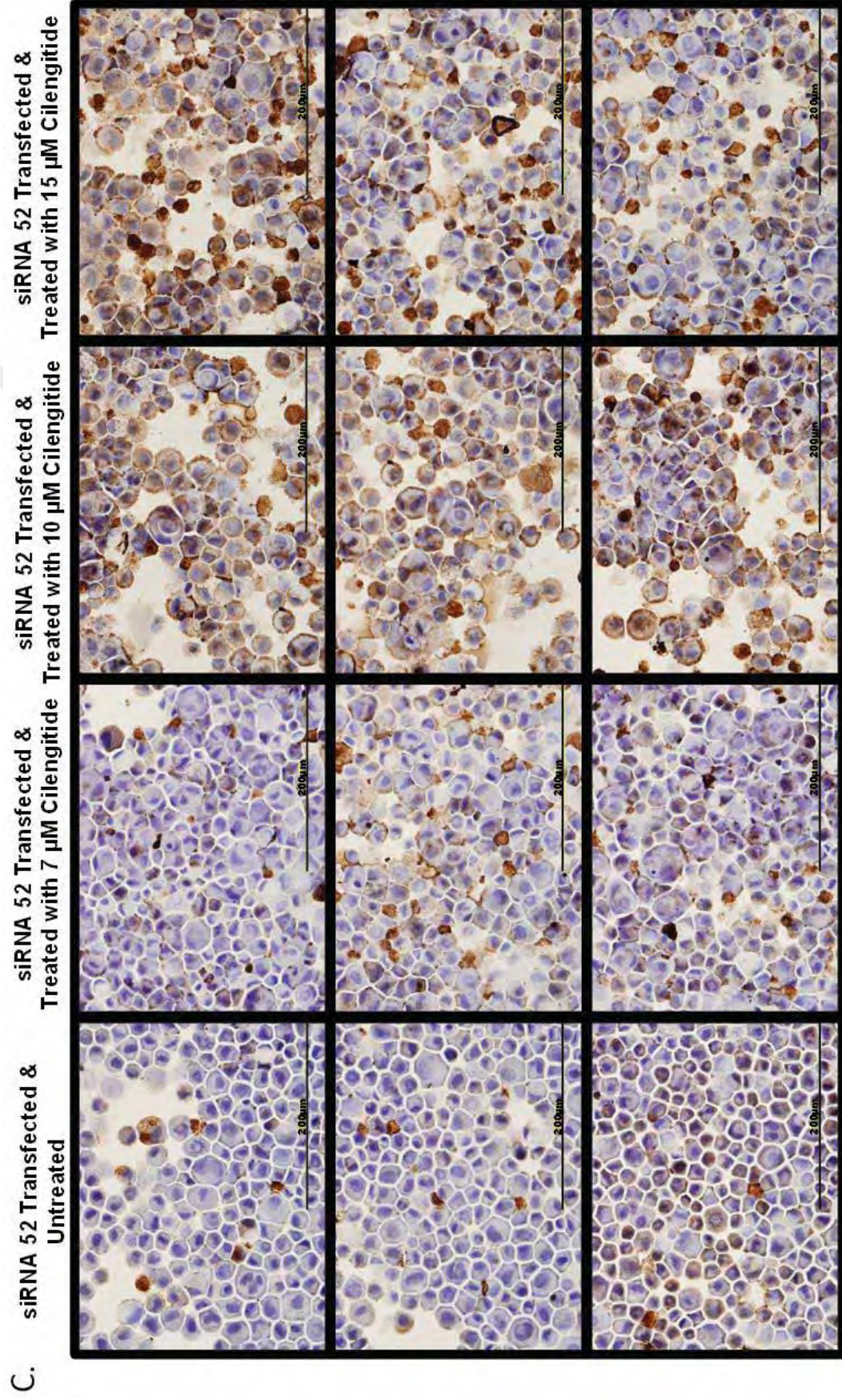
A.

Negative Control

Positive Control







D.

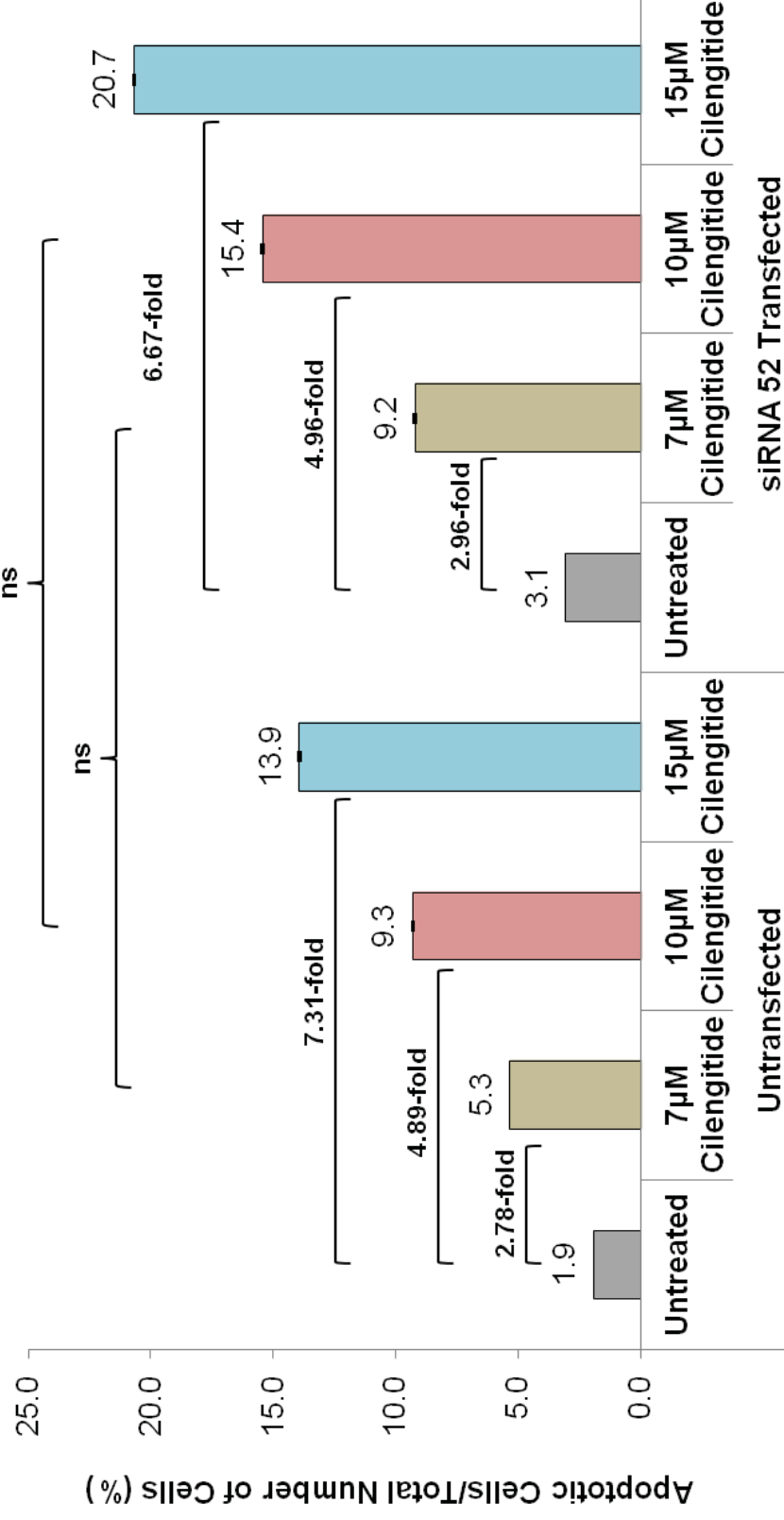


Figure 4.9 Dose-dependent increase in the number of apoptotic cells in untransfected and siRNA-52 transfected A172 cells stained with TUNEL (at 24 hr after addition of cilengitide)

(A) The yellow arrows indicate TUNEL-positive apoptotic cells (brown) in positive control. The blue-stained cells (haematoxylin staining) were considered non-apoptotic, 40X magnification. **(B)** Representative images of treatment-free untransfected and siRNA-52 transfected A172 cells, 40X magnification. **(C)** Representative images of untransfected and siRNA-52 transfected A172 cells treated with 7, 10 and 15 μ M of cilengitide, 40X magnification. **(D)** The percentage of apoptotic cells following 24 hr treatment with 7 μ M cilengitide shown in bar graph.

Results were normalised to untreated control groups of each untransfected and transfected cell groups. Statistical significance of differences was calculated relative to untransfected cell groups in each cilengitide treatment group. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

4.4 DISCUSSION

In this chapter, we have examined the relationship between the expression levels of NG2 and its potential impact on drug resistance. Cilengitide selectively targets $\alpha v\beta 1$ and $\alpha v\beta 5$ integrins and blocks the activation of crucial downstream signaling molecules. NG2 co-localises with integrin heterodimers containing B1 subunits. As initially hypothesised, cells expressing high levels of NG2 was associated with resistance to drug treatments, due to important contribution of NG2 to increased motility, survival and proliferation of cancer cells. Therefore, knock-down of NG2 expression in the cell line expressing high levels of NG2 was thought to alter aggressiveness of the glioma cells. Combination of NG2 knock-down with cilengitide treatment was therefore considered to show a collective impact on survival and proliferation of glioma cells. The process is schematically summarised in Figure 4.10.

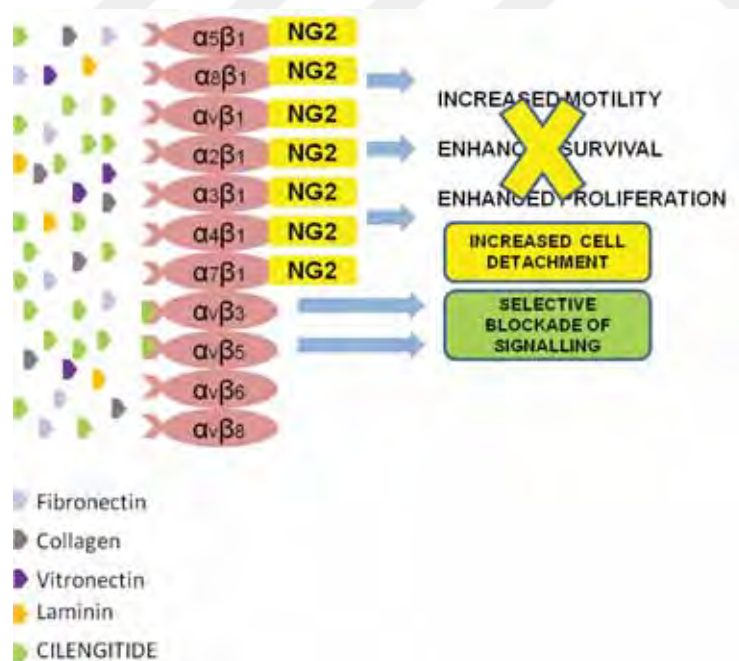


Figure 4.10 Summary of hypothesised association between NG2, cilengitide and integrins

High mRNA and protein expression of NG2 was detected in A172 and U-87 MG cell lines, while T98G, M059K and M059J cell lines demonstrated low levels of NG2 expression. Chekenya and colleagues reported high endogenous expression levels of NG2 in A172 and U-87 MG cell lines while no expression of NG2 was detected in the U251 cell line which confirms variable NG2 expression in glioma cell lines (Chekenya et al., 2008).

Glioma cell lines, A172, U-87 MG and osteosarcoma cell line MG-63 were relatively sensitive to cilengitide compared to T98G, M059K and M059J. Interestingly, cilengitide treatment of the cell lines revealed an unexpected response pattern when correlated with NG2 expression levels of each cell line. NG2 positive control cell line MG-63 and glioma cell lines expressing high levels of NG2 were found to be more susceptible to cytotoxic effects of the cilengitide. In contrast, a study demonstrated an association of high NG2 expression with increased chemoresistance to etoposide and vincristine (Chekenya et al., 2008). The same researchers also reported higher sensitivity to doxorubicin, etoposide and carboplatin in glioma spheroids high in NG2 (Chekenya et al., 2008). In addition, a role for NG2 in radioresistance has been shown in a study using U251, U251-NG2 transfected and U-87 MG cell lines (Hovinga et al., 2005). However, it has been suggested that U-87 MG cell line expressing NG2 endogenously resulted in significantly higher radioresistance, while U251 and U251-NG2 transfected cell lines demonstrated no difference between their degree of resistance to radiotherapy (Hovinga et al., 2005).

Cilengitide-resistant cell lines, T98G, M059K and M059J were also established as resistant to etoposide treatment as extensively discussed in Chapter 3. In addition, dose-response curves of A172 and U-87 MG cell lines produced significantly lower IC₅₀ values for etoposide compared to T98G, M059K and M059J. One possible explanation for the conflict between the findings of our study and present limited literature could be due to contribution of additional factors. Although an extensive literature exists regarding the functions of integrins in increased cell growth and chemoresistance (De et al., 2005; Desgrosellier and Cheresch, 2010; Weis and Cheresch, 2011), down-regulation of some integrin complexes such as integrin $\beta 1$, $\alpha 6$ and $\beta 4$ have been reported to contribute to resistance of the tumour cells to chemotherapeutic agents (Gingras et al., 1995; Kim et al., 2011a; Previtali et al., 1996). In addition, high integrin $\beta 3$ expression has been shown to correlate with increased sensitivity of U-87 MG, U373MG and T98G cells to BCNU and etoposide (Kim et al., 2011a). It may be proposed that multifaceted function of the members of the large integrin family could potentially have a role in response of glioma cells to chemotherapeutic agents. Although it is not the main aim of this study, the lack of analysis of expression levels of a panel of integrin complexes including $\alpha 3\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 4\beta 1$ associated with cilengitide response or NG2 co-localisation can be noted as one limitation of the present study. An extended study involving analysis of other factors may facilitate the acquisition of more informative data in understanding the cross-talk between NG2 and other effectors of chemotherapy response.

NG2 expression in A172 cells was suppressed using NG2 siRNA to establish whether increased response to cilengitide is a general response of GBM cells or associated with NG2 expression levels. Although a slight increase in cell death was observed in

siRNA-52 transfected and cilengitide-treated (7 μ M, IC₅₀ for A172) cells, the difference was not found to be statistically significant (see Figure 4.7, C). All cell groups, transfected with NG2 siRNA-52, a negative control Allstars siRNA nucleofection reagent and untransfected cells indicated a-dose dependent detachment of the cells by increasing cilengitide concentrations. This finding was consistent with a number of studies which have previously demonstrated the effect of cilengitide on detachment of endothelial cells (Alghisi et al., 2009; Oliveira-Ferrer et al., 2008) and glioma cell lines (Maurer et al., 2009; Oliveira-Ferrer et al., 2008). Cilengitide has been shown to exert its inhibitory effects through blocking binding of ECM components to α v β 3 and α v β 5 integrins which in turn impedes signalling to downstream effectors such as Akt and FAK (Oliveira-Ferrer et al., 2008; Smith, 2003).

The effect of cilengitide (7 μ M) in inducing apoptosis of the untransfected and siRNA-52, Allstars negative control and nucleofection reagent transfected A172 cells was determined by TUNEL staining. Results showed that cilengitide significantly triggered apoptosis of untransfected A172 cells compared to untreated group. Untransfected and siRNA-52 transfected cells were also exposed to higher doses of cilengitide and treatment with cilengitide and all doses examined, 7, 10 and 15 μ M, increased the number of apoptotic cells in untransfected A172 cells at slightly higher rates when each group was compared with untreated control cells. When the results obtained from each drug concentration was compared with each other, the apoptosis rate of A172 cells indicated no significant differences between untransfected and siRNA-52 transfected groups. Our results were consistent with the findings of previous studies showing cilengitide-caused detachment and subsequently cilengitide-initiated apoptosis of the cells (Lomonaco et al., 2011; Maubant et al., 2006).

To conclude, the results of this study showed that glioma cell lines express variable levels of NG2. In the present study there is also evidence that glioma cell lines differ in their response to cilengitide treatment. Although no contribution of NG2 overexpression to chemoresistance was documented, a potential opposite relationship was investigated. Because of the fact that suppression of NG2 expression levels did not alter cilengitide response of the cell lines considerably, the function of NG2 in signalling pathways associated with response to chemotherapeutics remains unclear.



CHAPTER 5

THE ROLE OF MGMT PROMOTER METHYLATION AND LEVEL OF PROTEIN EXPRESSION IN DETERMINING THERAPY RESPONSE AND SURVIVAL OUTCOME

5.1 INTRODUCTION

5.1.1 Temozolomide (TMZ)

TMZ [8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4-(3*H*)-one] (CCRG 81045) was first synthesised by Stevens and colleagues at Aston University, UK (Stevens et al., 1987). TMZ is an orally administered chemotherapy drug that can penetrate the blood brain barrier (BBB) with rather low toxicity with high bio-availability (Payne et al., 2005). It was first tested in the malignant brain tumour treatment in the early 1990s and a phase II study showed that 24% of patients were responsive to the TMZ therapy; this result led researchers to further investigate efficacy of TMZ as a glioma therapy (Newlands et al., 1996; Oreilly et al., 1993). TMZ is spontaneously converted to its active form 3-methyl-(triazene-1-yl)imidazole-4-carboxamide (MTIC) at physiological

pH in aqueous solution (Denny et al., 1994). A variety of methyl adducts; N⁷-methylguanine (~70%), N³-methyladenine (~10%) and O⁶-methylguanine (~9%) are generated by active metabolite MTIC which reacts with guanine residues in DNA (Tentori and Graziani, 2002). Although N⁷-methylguanine methyl adducts form the majority of total DNA adducts, methyl adducts at O⁶ position of guanine are more toxic than N⁷-methylguanine adducts and they are considered to be primarily responsible for cytotoxic effects of TMZ (Tentori and Graziani, 2002). The presence of DNA O⁶-methylguanine adducts lead to the generation of mismatches with cytosine or thymine which ultimately triggers apoptosis of the cells (Meikrantz et al., 1998).

5.1.2 MGMT

The *MGMT* gene resides on chromosome 10q26 (Natarajan et al., 1992). The gene contains five exons and four introns which span more than 170 kb (Nakatsu et al., 1993). A small protein with a molecular weight of 23 kDa consisting of 207 amino acids is the translation product of *MGMT* gene. Translocation of MGMT from cytoplasm to nucleus is facilitated by cellular factors and DNA binding domain of the protein maintains its ability to bind DNA elements in nucleus. (Pegg et al., 1995). MGMT removes alkyl adducts at the O⁶ position of DNA-guanine generated by DNA alkylating chemotherapy drugs and acts by preventing the formation of DNA double strand breaks (Kaina et al., 2007). The alkyl group is transferred to internal cysteine residue which locates at position 145 in the human MGMT. Interaction of MGMT with a substrate gives rise to irreversible inactivation of the enzyme (Pegg, 2000). Inactive MGMT is ubiquitinated and undergoes further degradation through the ATP-dependent protease complex (Srivenugopal et al., 1996). Overexpression of MGMT by tumour cells usually provides a poor prognosis as demonstrated in patients with malignant astrocytoma treated with bis-chloroethylnitrosourea (BCNU) (Jaeckle et al., 1998) and newly

diagnosed glioblastoma treated with TMZ (Friedman et al., 1998). In general, suppressed expression of MGMT is found to be correlated with methylated *MGMT* promoter and lack of MGMT promoter methylation is linked to resistance to TMZ in glioma patients (Hegi et al., 2004; Hegi et al., 2005; Paz et al., 2004). Potential prognostic value of MGMT and its role in chemoresistance are extensively discussed in Sections 1.1.4.3 and 1.1.5.3 in Literature Review, Chapter 1.

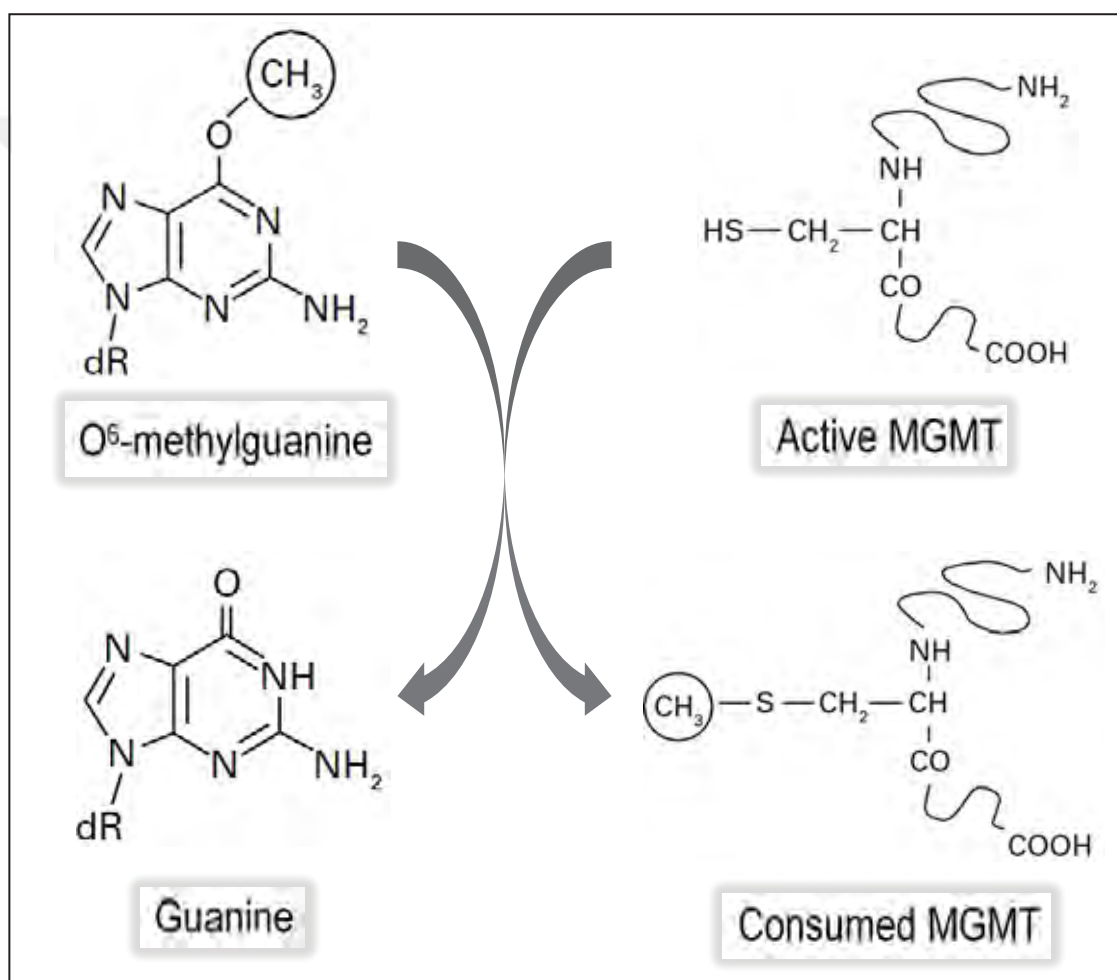


Figure 5.1 The role of MGMT in DNA repair (Hegi et al., 2008)

The O⁶ methyl group of guanine is removed by O⁶-methylguanine methyl transferase (MGMT). The function of this enzyme is to transfer the methyl group from the O⁶-methylguanine to the cysteine residue in the enzyme which results in irreversible inactivation of MGMT.

Working hypothesis of this chapter:

The response of cell lines to TMZ and patient response to standard treatment including chemotherapy with TMZ is determined by the methylation status of *MGMT* promoter. There is a direct relationship between methylated *MGMT* promoter, decreased expression levels of MGMT protein and better response to TMZ.

The general aim of this chapter is to assess the correlation of methylation status of *MGMT* promoter in glioma cell lines and tumour samples from glioblastoma patients treated with response to TMZ.

The specific aims of the chapter as follows:

1. To assess cellular response of five glioma cell lines to TMZ by measuring viability of cells using MTS cell proliferation assay.
2. To measure *MGMT* promoter methylation status in the cell lines and 42 glioblastoma tumour samples using both MSP and a quantitative *MGMT* promoter methylation analysis method, pyrosequencing.
3. To measure endogenous protein expression levels of MGMT in vitro and in clinical samples by immunoblotting.
4. To investigate the relationship between *MGMT* promoter methylation, protein expression and response characteristics of glioma cell lines.
5. To correlate results of promoter methylation and protein expression of MGMT with the response and overall survival in 42 glioblastoma patients.

5.2 METHODS

5.2.1 Ethics

Human research ethics approval for the use of fresh frozen tumour specimens was granted on 19 October 2009 (protocol 0911-303M) by The HAWKESBURY Human Research Ethics Committee (HREC) of Northern Sydney Central Coast Health (NSCCH).

5.2.2 Clinical Data

Clinical data collected included patient's age, gender, diagnosis, vital status, type of surgery, number of surgeries, administered concurrent/adjuvant and salvage therapies. The clinical characteristics of patients whose tumours were studied in this thesis are detailed in Table 5.1. It should be noted that this patient cohort (training) included 37 male and 5 female individuals, a male:female ratio of 7.4:1.

5.2.3 Classification of Samples

For the analysis of response outcome two endpoints were defined. Primary endpoint was 6 months of progression free survival (PFS6) and defined as the time from treatment initiation until tumour progression. A secondary endpoint of overall survival (OS) was used and calculated from the date of diagnosis until the date of death. OS was used as a secondary analysis point because patients will have received additional salvage therapies at recurrence after RT/TMZ, The census date of 01.09.2010 was used for patients who were alive (n = 4). The median overall survival of our cohort (13.8 months) was used to classify patients into two survival groups. Samples were then assigned to the sample groups of 'responders' (n = 15) or 'non-responders' (n = 12) for response cohort and long term survivors (LTS) (n = 19) and short term survivors (STS) (n = 19) for survival cohort (see Table 6.4, Chapter 6).

Table 5.1 Clinical data of patients whose tumours were included in biomarker discovery study using SELDI-TOF MS

Tumour Bank Code	Gender	Diagnosis	Status	Age (year)	Concurrent Therapy	Salvage Therapy
B30051-08	M	GLIOMA	ALIVE	60	TMZ/RT	TMZ
B3380-06	M	GLIOMA	DEAD	88	TMZ/RT	TMZ
B3855-07	M	GLIOMA	DEAD	75	TMZ/RT	TMZ
B3852-07	M	GLIOMA	DEAD	48	TMZ/RT	TMZ+Procarbazine
B3850-07	M	GLIOMA	DEAD	75	TMZ/RT	TMZ
B30043-08	M	GLIOMA	ALIVE	67	TMZ/RT	TMZ
B3944-07	M	GLIOMA	DEAD	52	TMZ/RT	TMZ
B3832-07	F	GLIOMA	DEAD	64	TMZ/RT	TMZ/Procarbazine, Cilengitide
B3447-06	M	GLIOMA	DEAD	74	TMZ/RT	TMZ
B3468-06	F	GLIOMA	ALIVE	64	TMZ/RT	TMZ
B3470-06	M	GLIOMA	DEAD	63	TMZ/RT	TMZ+Procarbazine, Carboplatin, Avastin
B3496-07	M	GLIOMA	DEAD	50	TMZ/RT	TMZ, Procarbazine, Carboplatin, Thalidomide
B3812-07	M	GLIOMA	DEAD	51	TMZ/RT	TMZ
B3829-07	M	GLIOMA	DEAD	71	TMZ/RT	Carboplatin
B3847-07	M	GLIOMA	DEAD	63	TMZ/RT	TMZ, Carboplatin+Etoposide+Cilengitide
B3880-07	M	GLIOMA	DEAD	58	TMZ/RT	TMZ+Procarb, Carboplatin, Avastin
B3925-07	F	GLIOMA	DEAD	78	TMZ/RT	TMZ
B3936-07	M	GLIOMA	DEAD	63	TMZ/RT	Procarbazine, TMZ
B3982-08	M	GLIOMA	DEAD	47	TMZ/RT	Avastin, Procarbazine+TMZ
B3887-07	M	GLIOMA	DEAD	44	TMZ/RT	TMZ, Carboplatin/Etoposide/Thalidomide
B3490-07	M	GLIOMA	DEAD	69	TMZ/RT	TMZ
B30089-08	M	GLIOMA	DEAD	49	TMZ/RT	TMZ
B30025-08	M	GLIOMA	DEAD	67	TMZ/RT	TMZ+Procarbazine, Cilengitide
B30073-08	M	GLIOMA	DEAD	73	TMZ/RT	TMZ
B30062-08	M	GLIOMA	DEAD	31	TMZ/RT	TMZ
B30070-08	M	GLIOMA	DEAD	64	TMZ/RT	TMZ, Carboplatin+Etoposide
B3808-07	M	GLIOMA	DEAD	76	TMZ/RT	TMZ
B3858-07	M	GLIOMA	DEAD	69	TMZ/RT	TMZ
B3488-07	M	GLIOMA	DEAD	67	TMZ/RT	TMZ
B30049-08	M	GLIOMA	DEAD	83	TMZ/RT	TMZ
B3495-07	F	GLIOMA	DEAD	67	TMZ/RT	TMZ
B3489-07	M	GLIOMA	DEAD	64	TMZ/RT	TMZ
B3971-08	M	GLIOMA	DEAD	59	TMZ/RT	TMZ
B3457-06	M	GLIOMA	DEAD	75	TMZ/RT	TMZ
B3861-07	F	GLIOMA	DEAD	75	TMZ/RT	TMZ, Procarbazine+Avastin+Cilengitide, Carboplatin+Etoposide
B3860-07	M	GLIOMA	DEAD	58	TMZ/RT	TMZ+Procarbazine, Cilengitide, Avastin
B30030-08	M	GLIOMA	DEAD	57	TMZ/RT	TMZ+Procarbazine
B3980-08	M	GLIOMA	DEAD	65	TMZ/RT	Carboplatin, Carboplatin+Avastin, Carboplatin+Etoposide
B3967-08	M	GLIOMA	DEAD	70	TMZ/RT	TMZ
B3947-07	M	GLIOMA	DEAD	72	TMZ/RT	TMZ
B3814-07	M	GLIOMA	DEAD	53	TMZ/RT	TMZ
B3881-07	M	GLIOMA	DEAD	64	TMZ/RT	TMZ

Abbreviations: F: female, M: male, RT: radiotherapy, TMZ: temozolomide

5.2.4 Preparation of TMZ

Temozolomide capsules (100 mg; TMZ; Temodar®, Schering-Plough Corporation, Kenilworth, NJ, USA) were kindly donated by Dr Helen Wheeler (oncologist, Northern Cancer Clinic, North Shore Private Hospital) when the drug was in excess. TMZ stock solution was prepared by dissolving the entire capsule's contents in DMSO (volume). The maximum soluble amount of temozolomide in DMSO was 90 mM. Dilutions of 42µM to 1000 µM were used for chemosensitivity assays. Freshly dissolved TMZ solution was added to the cell culture after sterilisation using 0.22 µm (pore size) filters. TMZ response was measured using MTS cell proliferation assay as described in Section 2.2.3, Materials and Methods, Chapter 2.

5.2.5 DNA Protocols for Extraction and Determination of MGMT Promoter Methylation

5.2.5.1 DNA Extraction from Cells

DNA extraction from cell lines was performed using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Cells were harvested as described in Section 2.2.1 and collected in a microcentrifuge tube. Pellets of a maximum number of 5×10^6 cells were resuspended in a final volume of 200 µL PBS. Proteinase K (Roche, Indianapolis, IN, USA) (20 µl) and AL buffer (200 µl) were added to the cell suspensions followed by pulse vortexing for 15 seconds to mix. The tubes were incubated at 56 °C for 10 minutes. Following this incubation step, 200 µl ethanol (96-100%) was added and tubes were mixed by vortexing, followed by a brief spin. The mixtures were then transferred onto a QIAamp Mini Spin Column and centrifuged at

6,000 x g for 1 minute. The spin column was transferred into a clean 2 ml collection tube and the tube containing the filtrate was discarded. Buffer AW1 (500 µl) was added to the spin column then centrifuged at 6,000 x g for 1 minute. The spin column was transferred onto a fresh collection tube and the filtrate was again discarded. Buffer AW2 (500 µl) was added to the spin column followed by centrifugation at 20,000 rpm for 3 minutes. The filtrate was again discarded and the spin column was transferred into a 1.5 ml microcentrifuge tube. 20 µl of buffer AE was transferred directly to the membrane of the spin column and incubated at room temperature for 5 minutes before DNA elution by centrifugation at 6,000 x g for 1 minute. Eluted DNA was then stored at 4 °C.

5.2.5.2 DNA Extraction from Paraffin Embedded Tumour Tissue

QIAamp® DNA FFPE Tissue Kit was used to extract DNA from Formalin-Fixed, Paraffin Embedded (FFPE) tissue sections. FFPE sections cut from sample blocks were obtained from Department of Anatomical Pathology, Royal North Shore Hospital, Sydney, Australia. FFPE sections were placed into centrifuge tubes and 100% xylene (1200 µl) was added to de-paraffinise the tissue (in a fume hood). Tubes were mixed by vortexing and incubated on rotating wheel for 20 minutes then centrifuged at full speed for 5 minutes at room temperature (15-25 °C). Supernatants were discarded and xylene was re-added (as before). 1200 µl of ethanol (96-100%) was added to the pellets and mixed by vortexing. After full speed centrifugation for 5 minutes at room temperature, ethanol was removed. The same amount of ethanol was added one more time and centrifugation was repeated. Centrifuge tubes were incubated for 15 minutes at 37 °C to allow any remaining ethanol to evaporate. The pellets were resuspended in buffer ATL (180µl) then proteinase K (40 µl) (Roche, Indianapolis, IN, USA) added and mixed by vortexing. The tubes were incubated at 56°C in a shaking water bath overnight. The following day Proteinase K solution was refreshed and the tubes were again re-

incubated at 56 °C in a shaking water bath overnight. Buffer AL (200 µl) was added to the samples, mixed by vortexing and followed by addition of 200 µl ethanol. The mixture was then vortex mixed and transferred to the QIAamp MiniElute column placed in a 2 ml collection tube then centrifuged at 6000 x g for 1 minute. The flow-through was discarded and the column transferred to a fresh collection tube. Buffer AW1 was added into the columns and centrifuged at 6000 x g for 1 minute before removing the flow-through. This step was repeated with Buffer AW2 and columns were transferred into clean collection tubes. The columns were centrifuged at 20000 x g for 3 minutes to remove any remaining liquid and placed in a 1.5ml microcentrifuge tube. Buffer ATE (20 µl) was added to the membranes and incubated at room temperature for 5 minutes then centrifuged at full speed for 1 minute. The eluents containing DNA were quantified using NanoDrop® ND-1000 (NanoDrop, Wilmington, DE, USA) spectrophotometer at the absorption A_{260}/A_{280} ratio before storage at 4 °C.

5.2.5.3 DNA Bisulfite Treatment

DNA was extracted from cell lines and paraffin embedded tumour tissue as described in Sections 5.2.5.1 and 5.2.5.2. Sample DNA was subjected to bisulfite treatment by using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) which converts unmethylated cytosine into uracil while methylated cytosine remain intact. After sodium bisulfite conversion, the methylation status of MGMT promoter was detected by methylation-specific PCR (MSP). Unmethylated DNA Epiect human control DNA (Qiagen, Hilden, Germany) and human HCT 116 DKO Methylated DNA Standard control DNA (Zymo Research Corp., CA, USA) were included in each set of the treatment. The sample concentrations were adjusted to 25 ng/µl with water. Total volume of 20 µl (500 ng) DNA sample were subjected to bisulfite treatment for each sample. Water (900 µl), M-Dilution Buffer (300 µl) and M-Dissolving Buffer (50 µl)

were added into one tube of CT Conversion Reagent prior to use. The tube was mixed by vortexing for 10 minutes. Final volume of 20 µl DNA sample was mixed with 130µl of CT Conversion Reagent in PCR tubes then placed in a thermal cycler and following steps were performed; 98 °C for 10 minutes, 64°C for 2.5 hours and hold at 4 °C. Bisulfite-converted DNA sample was added into a Zymo-Spin IC Column containing M-Binding Buffer (600 µl) and mixed by inverting the column several times. The column (placed in a collection tube) was centrifuged at full speed (>10,000 x g) for 30 seconds. The flow-through discarded and M-Wash Buffer (100 µl) was added to the column and again centrifuged at full speed for 30 seconds. For desulphonation, M-Desulphonation Buffer (200 µl) was added to the column, incubated for 15-20 minutes at room temperature then centrifuged at full speed for 30 seconds. The column was washed twice with M-Wash Buffer (200 µl) and transferred in a 1.5ml centrifuge tube. Bisulfite modified DNA was eluted from the column matrix with M-Elution Buffer (10 µl) and stored at -80 °C.

5.2.5.4 Methylation-Specific PCR (MSP)

Bisulfite treated DNA (see Section 5.2.5.3) was subjected to MSP analysis with primers specific for MGMT gene. Two rounds of PCR reactions were conducted using Platinum® *Taq* PCR DNA Polymerase (Invitrogen, Carlsbad, CA, USA) system. Unmethylated DNA Epitect human control DNA (Qiagen, Hilden, Germany) and human HCT 116 DKO Methylated DNA Standard control DNA (Zymo Research Corp., CA, USA) were included in each set of PCR reactions. Reaction mixtures were prepared for the first round of MSP as explained in Table 5.2 and placed into MJ DNA Engine, DYAD, Peltier Thermal Cycler (MJ Research, Waltham, MA). The reaction was performed as shown in Table 5.3. 1 µl of first round DNA product was diluted in Nuclease Free Water to a final volume of 50 µl. In the second round of MSP, 5 µl of the

diluted DNA sample were used in preparing PCR mixture in combination with other reagents as listed in Table 5.4. 40 PCR cycles were conducted as detailed in Table 5.5. DNA (19 µl) product was analysed in a 1.5% agarose gel as described in Section 5.2.5.6 and visualised using the Gel Doc XR Documentation System (Bio-Rad Lab., 2000 Alfred Nobel Dr., Hercules, CA, USA).



Table 5.2 MGMT PCR reagents

Reagents (PCR I)	Amount (µl/reaction)
10X PCR Amplification Buffer	2.5
10X PCR Enhancer Solution	2.5
MgSO ₄ (50 mM)	1
dNTPs (10 mM)	2
Forward Primer (20 µM)	0.625
Reverse Primer (20 µM)	0.625
Nuclease Free Water	15
Platinum Taq Polymerase	0.25
DNA Sample	1

Abbreviations: dNTPs: deoxynucleotide triphosphates, MgSO₄: magnesium sulphate, PCR: polymerase chain reaction

Table 5.3 MGMT thermal cyclor conditions

Steps	Temperature (PCR I)
1	95 °C for 10 minutes
2	95 °C for 30 seconds
3	52 °C for 30 seconds
4	72 °C for 30 seconds
5	Step 2-4 repeated 40 cycles
6	72 °C for 10 minutes
7	4 °C forever

Table 5.4 MGMT PCR reagents

Reagents (PCR II)		Reagents (PCR II)	
Methylated Primers	Amount (µl/reaction)	Unmethylated Primers	Amount (µl/reaction)
10X PCR Amp.	2.5	10X PCR Amp.	2.5
10X PCR Enhancer	2.5	10X PCR Enhancer	2.5
MgSO ₄ (50 mM)	1	MgSO ₄ (50mM)	1
dNTPs (10 mM)	2	dNTPs (10mM)	2
Forward Primer (20 µM)	0.625	Forward Primer (20 µM)	0.625
Reverse Primer (20 µM)	0.625	Reverse Primer (20 µM)	0.625
Nuclease Free Water	15	Nuclease Free Water	15
Platinum Taq Polymerase	0.25	Platinum Taq Polymerase	0.25
DNA Sample	5 (diluted product)	DNA Sample	5 (diluted product)

Abbreviations: Amp.:amplification buffer, dNTPs: deoxynucleotide triphosphates, MgSO₄: magnesium sulphate, PCR: polymerase chain reaction

Table 5.5 MGMT thermal cycling conditions

Steps	Temperature (PCR II)
1	95 °C for 10 minutes
2	95 °C for 15 seconds
3	52 °C for 15 seconds
4	72 °C for 15 seconds
5	Step 2-4 repeated 40 cycles
6	72 °C for 10 minutes
7	4 °C forever

5.2.5.5 Pyrosequencing

MGMT promoter pyrosequencing was performed using the PyroMark Q96 CpG MGMT kit (Qiagen, Hilden, Germany). DNA was bisulfite converted and PCR reaction mixture (50 µl) was prepared using the reagents detailed in Table 5.6 and run on thermal cycler with the conditions listed in Table 5.7. Streptavidin sepharose beads (3 µl) and pyrosequencing binding buffer (37 µl) (see Appendix B) were added to the PCR products (50 µl) and the mixture was incubated for 20 minutes on Eppendorf MixMate® (Eppendorf, Hamburg, Germany). Whilst incubating, mastermix of 0.4 µM sequence primer in annealing buffer was prepared and added into the appropriate wells of the pyrosequencing plate. The vacuum prep tool was prepared and PCR tubes were placed on vacuum to capture the beads and were washed with 70% ethanol, denaturation solution (see Appendix B) for 5 seconds and wash buffer (see Appendix B) for 10 seconds. Vacuum prep tool was then turned off and transferred to the wells of the pyrosequencing 96 well plate pre-filled with mastermix. The prep tool was then washed with MilliQ water for 15 seconds. For the pyrosequencing reaction, the plate was heated at 90 °C for 2 minutes to denature the template. Instrument parameters were selected according to the manufacturer's instructions and the cartridge was loaded with substrate, enzyme and dNTPs. The plate was placed in the PSQ 96 instrument and the cartridge was inserted thoroughly. PCR products were further analysed by pyrosequencing and the following sequence was entered into the PyroMark™ software program version 2.0.4 (Qiagen, Hilden, Germany).

‘T/CGTTTTGT/CGTTTT/CGAT/CGTTT/CGT/CAGG’ (Qiagen, Hilden, Germany)

Programs were produced and evaluated for sequence quality, and percent methylation at individual CpG sites was calculated using PyroMark™ software. The obtained methylation percentages were averaged over the five CpG loci tested. Samples were

considered methylated when the percentage of mean MGMT methylation value was $\geq 9\%$.

Table 5.6 MGMT PCR reagents for pyrosequencing

Reagents	Amount (μ l/reaction)
10X PCR Amplification Buffer	5
MgSO ₄ (50 mM)	1.5
dNTPs (10 mM)	1
Forward Primer (20 μ M)	1
Reverse Primer (20 μ M)	1
Nuclease Free Water	38
Platinum Taq Polymerase	0.5
DNA Sample	2

Abbreviations: MgSO₄: magnesium sulphate, PCR: polymerase chain reaction, dNTPs: deoxynucleotide triphosphates

Table 5.7 MGMT thermal cycler conditions for pyrosequencing

Steps	Temperature
1	95 °C for 10 minutes
2	95 °C for 30 seconds
3	50 °C for 30 seconds
4	72 °C for 30 seconds
5	Step 2-4 repeated 55 cycles
6	72 °C for 5 minutes
7	4 °C forever

5.2.5.6 Agarose (Horizontal) Gel Electrophoresis

MSP DNA products were separated on a 1.5% agarose (Ultrapure, Progen Industries Ltd., QLD, Australia) gel to evaluate methylation status of MGMT. The gel was prepared by addition of agarose 1.5% (w/v) in Tris-borate-EDTA (TBE) buffer (see Appendix B). The components were swirled to mix, weighed and heated until all agarose particles were dissolved then 10 µl of gel red was added to the solution. The mixture was allowed to cool about 60 °C at room temperature and poured into a horizontal gel electrophoresis tray containing a comb. The agarose mixture on the tray was allowed to solidify for 30 minutes. The comb was then removed and the tray was placed in an electrophoresis chamber. 0.5X TBE buffer was poured into the chamber until the gel was covered. Sample DNA (19 µl) was mixed with 10X BlueJuice Gel Loading Buffer (2 µl) (Invitrogen, Carlsbad, CA, USA) and loaded into the well. GeneRuler™ 1kb DNA Ladder (Thermo Scientific, Waltham, MA, USA) was included in each gel. The gel was electrophoresed for 30 min at 100 V and visualised with the Gel Doc XR Documentation System (Bio-Rad Lab., 2000 Alfred Nobel Dr., Hercules, CA, USA) using QuantityOne Software (Bio-Rad, Hercules, CA, USA).

5.2.5.7 Optimisation of PCR

Amounts of reagents were optimised using six different master mix combinations and determined with housekeeping gene β -Glucuronidase (*GUS- β*) amplification using primers (5'-3') *GUS- β 3'*: actatcgccatcaacaacacactcacc and *GUS- β 5'*: gacgggtgatgtcatcgtatgt. PCR master mix containing AmpliTaq Gold® DNA Polymerase (3000U) (Applied Biosystems, Foster City, CA, USA) was prepared for each sample as detailed in Table 5.8 and placed into the MJ DNA Engine, DYAD, Peltier Thermal Cycler (MJ Research, Waltham, MA) then, amplified using the program detailed in Table 5.9. No template controls were performed for each master mix combination. The

optimal master mix combination was chosen following assessment of PCR products using polyacrylamide gel electrophoresis as described in Section 5.2.5.6. Master mix 6 which produced the strongest band of *GUS-β* DNA product was selected for further use (see Figure 5.2). The remainder of PCR products were stored at 4 °C.

Table 5.8 Master Mix combinations for PCR optimisation

Reagents	1	2	3	4	5	6
Nuclease Free Water	36	27.5	35.5	36	38.5	36
10X PCR buffer II	5	5	5	5	5	5
dNTPs (2.5 mM, diluted from 100 mM)	2	1	2	1	1	1
GUS-β3'	1.25	1	1	1.25	1	1
GUS-β5'	1.25	1	1	1.25	1	1
MgCl ₂ (25 mM)	3	3	4	4	2	2
AmpliTaq Gold® DNA Polymerase (3000 U)	0.5	0.5	0.5	0.5	0.5	0.5
DNA Sample	1	1	1	1	1	1

Abbreviations: dNTPs: deoxynucleotide triphosphates, *GUS-β*: β-Glucuronidase, MgCl₂: magnesium chloride, U: units

Table 5.9 Thermal cycler conditions for *GUSβ* DNA amplification

Steps	Temperature
1	94 °C for 5 minutes
2	94 °C for 30 seconds
3	60 °C for 30 seconds
4	72 °C for 1 minute
5	Step 2-4 repeated 29 cycles
6	72 °C for 10 minutes
7	11 °C forever

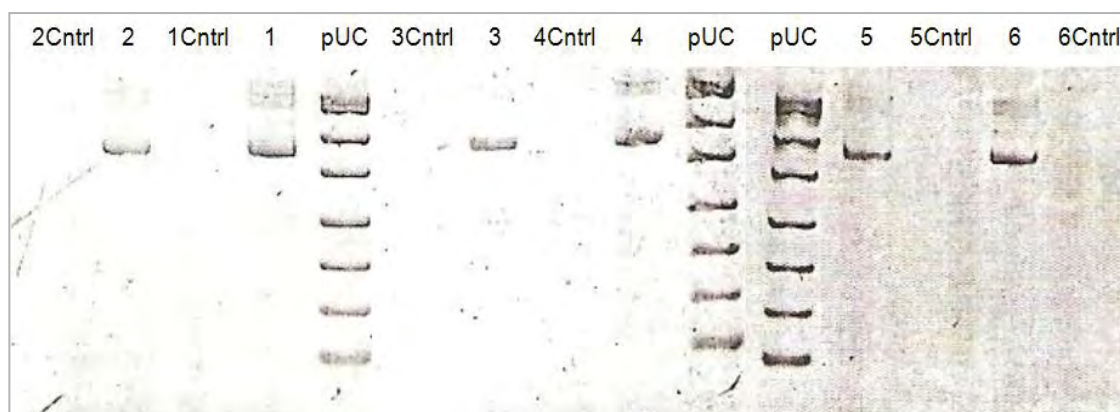


Figure 5.2 Polyacrylamide gel electrophoresis of PCR products

5.2.5.8 Vertical Polyacrylamide Gel Electrophoresis

Glass plate sandwich was assembled 1.56 ml of acrylamide:bisacrylamide 19:1 ultra pure (Amresco, Solon, Ohio, USA), 120 μ l of 10% Ammonium per sulphate (APS) (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) and 22 μ l of *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) were added to 15 ml of 0.5X TBE buffer (see Appendix B) then mixed by inverting 10 times. The mixture was instantly poured into an assembled glass plate sandwich and a comb was placed in the top of the solution. After a 30 minute polymerisation, the glass plates containing the acrylamide gel were placed in an electrophoresis tank loaded with 0.5X TBE buffer. Samples were prepared by combining PCR product (2 μ l) and Loading Buffer 6X (2 μ l), and then 500 bp DNA marker pUC19 DNA/MspI(HpaII) Marker (MBI Fermentas, Vilnius, Lithuania) and the samples were loaded into the wells. Electrophoresis was performed at 120 V for 35 minutes and the gel was stained with SYBR® Safe DNA gel stain for 20 minutes (Invitrogen, Eugene, OR, USA) and imaged with Fujifilm FLA 3000 Fluorescent Image Analyser (Fuji Photo Film Co. Ltd., Tokyo, Japan).

5.2.6 Immunoblotting for MGMT Protein

Protein from the cell lines and tumour tissues (n = 42) was extracted using a urea lysis buffer. Protocols for protein extraction in urea buffer are detailed in Section 6.2.1, Chapter 6. MGMT protein expression levels were detected using an anti-human, MGMT clone MT3.1 mouse monoclonal antibody (Millipore Corporation, Billerica, MA, USA). The protocols used to perform immunoblotting are described in more detail in Section 2.5.3, Material and Methods, Chapter 2.

5.2.7 Statistical Analysis

Statistical analysis (student t-test) of the data was performed using GraphPad Prism software (v.4, GraphPad Software, Inc.). Two tailed-Fisher's exact test was used to calculate significance between the methods used for detecting methylation (v.4, GraphPad Software, Inc.). Asterisk rating system (* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001) were used when representing significance. The correlations were calculated with Spearman's correlation coefficient using the SPSS statistical package, Version 18.0 (SPSS Inc, Chicago, IL). The correlation coefficients with p-value < 0.05 were considered significant.

5.3 RESULTS

5.3.1. Optimisation Steps to Prepare a Soluble and the Most Effective Form of TMZ for Cytotoxicity Assays

TMZ was a difficult drug to reconstitute. The processes for dissolving TMZ and determining optimal conditions for *in vitro* use of TMZ are summarised in Table 5.10. Table 5.11 lists different solvents, the final concentrations of TMZ solutions and results obtained from each attempt. TMZ was insoluble in milliQ water and PBS solutions. An improved dissolution rate of TMZ was obtained through increasing DMSO concentrations and complete dissociation of the drug particles was attained in 100% DMSO. Final concentration of TMZ starting from 5 mM to 120 mM was tested and the maximum soluble concentration of TMZ was determined to be 90 mM (see Table 5.12).

The effect of sterilisation using a 22 μ m (pore size) filter was assessed in four glioma cell lines; T98G, A172, M059K and U-87 MG. Figure 5.3 demonstrates the untreated control and only-DMSO treated control groups as well as filtered and non-filtered TMZ test groups treated for 48 hr with 66 μ M TMZ which was prepared from a 30mM stock solution of TMZ. A significantly higher cytotoxic effect of TMZ was noted for filtered TMZ groups of A172 and M059K compared to non-filtered TMZ treated groups. While treatment with both non-filtered and filtered TMZ groups resulted in significantly decreased cell viability in U-87 MG cells, T98G cells were equally resistant to both TMZ treatments. Overall, filter-sterilisation of TMZ solution was found to be significantly more effective than non-filtered TMZ solution in A172 and M059K cell lines.

Table 5.10 Optimisation steps of TMZ used in cytotoxicity assays to determine IC₅₀ dose of glioma cell lines

Applications	Results
1. Selection of Solvent to dissolve TMZ powder	DMSO
2. Effect of sterilisation method (filtration through 0.22 µm filter)	No effect
3. Minimising toxic effect of DMSO on cells by preparing more concentrated stock	90mM stock used
4. Optimal time point to collect IC ₅₀ for each cell line	96 hr

Abbreviations: DMSO: dimethyl sulfoxide, IC₅₀: half maximal inhibitory concentration, TMZ: temozolomide

Table 5.11 Solubility of TMZ in different concentrations of solvents

Solvent	Final Concentration (Active TMZ)	Result
PBS (without Ca ²⁺ , Mg ²⁺)	5 mM	Not completely dissolved
MilliQ water	5 mM	Not completely dissolved
0.1% DMSO	5 mM	Not completely dissolved
1% DMSO	5 mM	Not completely dissolved
10% DMSO	5 mM	Not completely dissolved
50% DMSO	5 mM	Not completely dissolved
100% DMSO	5 mM	Dissolved
100% DMSO	30 mM	Dissolved
100% DMSO	60 mM	Dissolved
100% DMSO	90 mM	Dissolved
100% DMSO	120 mM	Not completely dissolved

Abbreviations: DMSO: dimethyl sulfoxide, PBS: phosphate buffered saline, TMZ: temozolomide

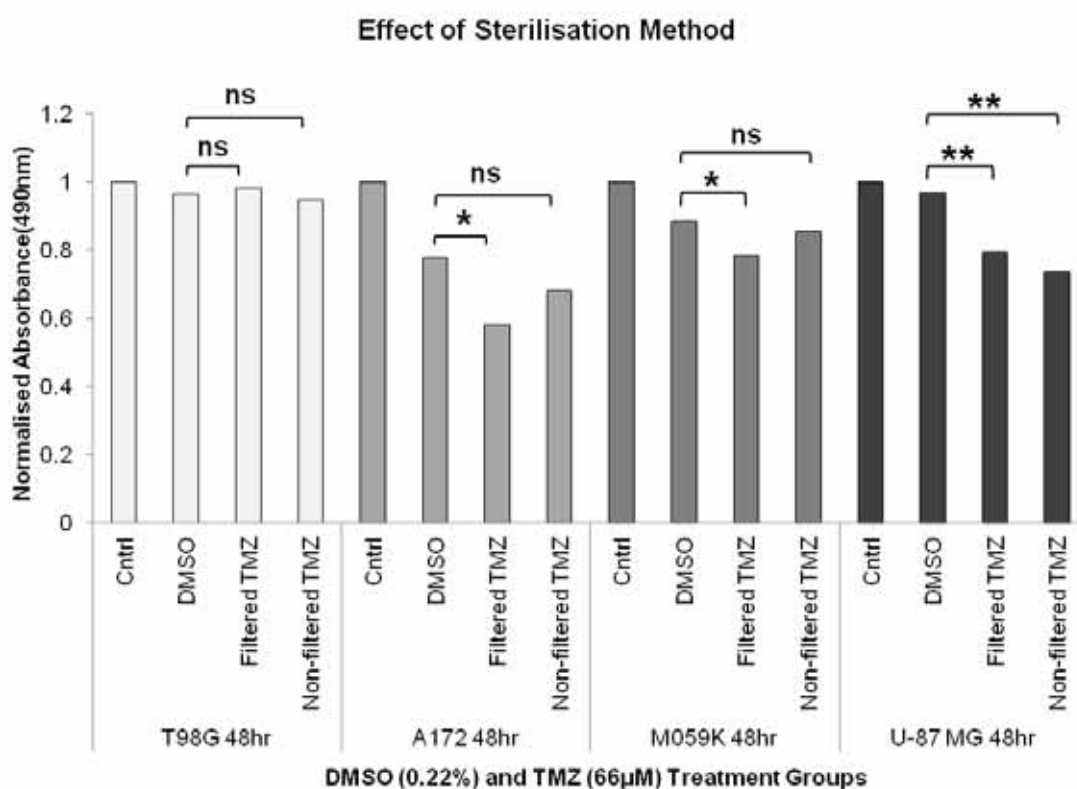


Figure 5.3 Effect of the sterilisation method on the effectiveness of drug

TMZ was filter sterilised through 0.22 µm (pore size) filters. MTS assay was performed to measure the effect of TMZ on four different glioma cell lines. Stock concentration of TMZ used in this assay was 30mM. Statistics were calculated relative to DMSO control.

ns: non-significant, *: p-value < 0.05, **: p-value < 0.01.

Abbreviations: Cntrl: Untreated Control Group, DMSO: dimethyl sulfoxide, TMZ: temozolomide

Cells were treated with TMZ doses (stock 30mM) from 42 to 1000 μ M and controls were treated with an equivalent percentage of DMSO in medium. Cell viability decreased steeply when the concentration of TMZ was increased to 500 and 1000 μ M in all cell lines. However, viability of cells was also reduced up to 31% at 1.66% dilution and more than 50% at 3.33% dilution of DMSO in DMSO control groups (see Figure 5.4). Among the five glioma cell lines, M059K cell line was the most sensitive to DMSO concentrations.

A more concentrated (60 mM) stock solution of TMZ was prepared to reduce percentage of DMSO in drug dilutions and eliminate DMSO interference on the cytotoxic effect of TMZ while drug treatment doses were remained unchanged. TMZ was again used at concentrations in the range 42 to 1000 μ M. Doubling TMZ concentration in stock solution lowered the DMSO percentage by 50% in each drug dilution (see Figure 5.5). Therefore, viability of the cells were improved for all cell lines (shown in Figure 5.5) when compared with the results of drug dilutions containing the same amount of TMZ but prepared using 30 mM TMZ stock (shown in Figure 5.4). However, only-DMSO control groups showed that 1.66% final DMSO percentage in drug solutions were highly toxic for M059K cells. While the viability of cells was decreased dramatically by 68% in M059K cells, other glioma cell lines showed a relatively better survival rate (up to 39% cell mortality) at the drug concentration of 1000 μ M containing 1.66% DMSO. Under these circumstances, none of the cell lines reached the IC₅₀ concentration of TMZ with an acceptable level (the percentage of viable cells greater than 80%) of cell viability in DMSO control groups (see Figure 5.5).

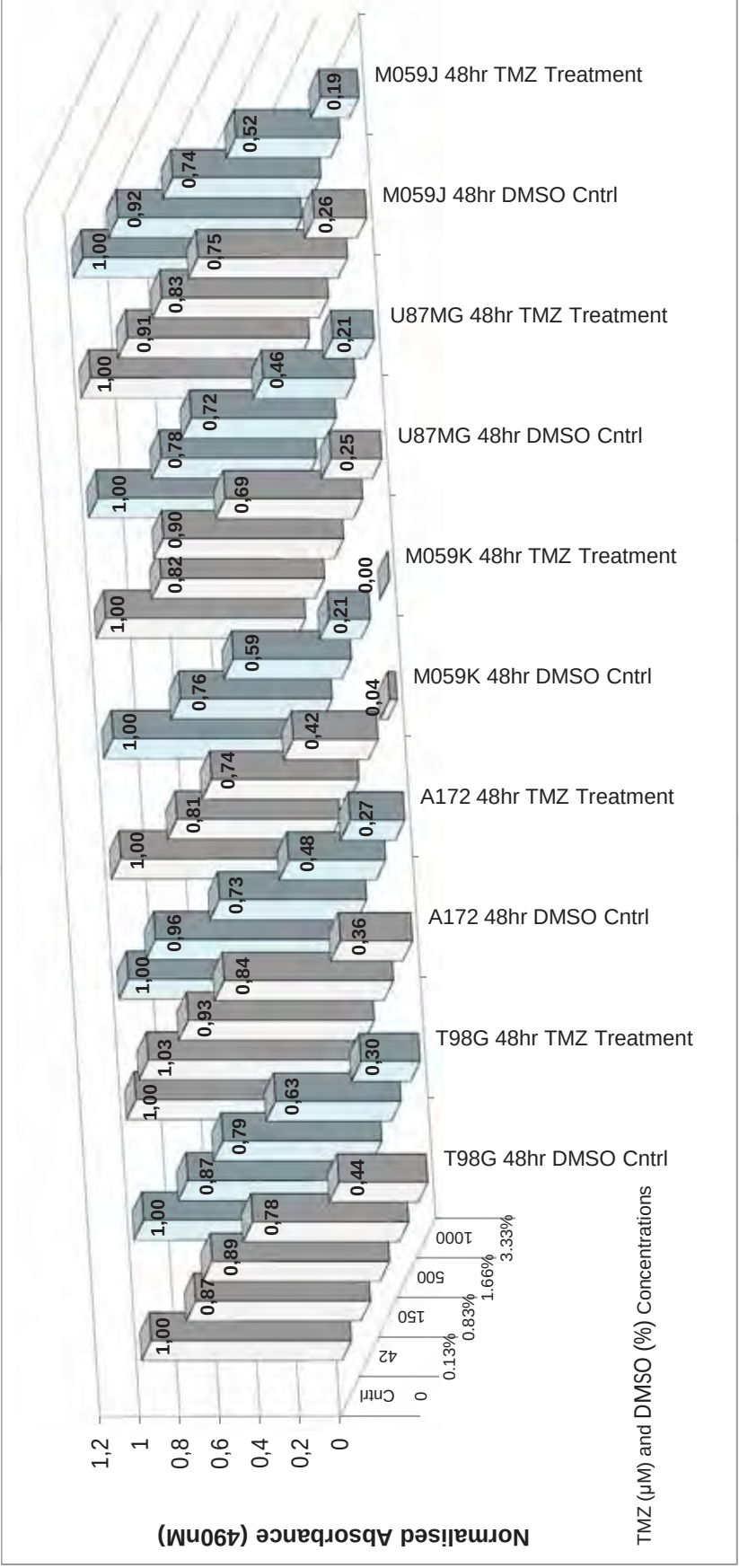


Figure 5.4 Response of glioma cell lines at 48 hr after TMZ treatment (a stock concentration of 30mM TMZ)

The viability of untreated, DMSO-only and TMZ treatment groups of five glioma cell lines. The main stock of TMZ was prepared in 100% DMSO, therefore, each drug dilution contains different percentages of DMSO. Bars represent relative number of total viable cells.

Abbreviations: Cntrl: Untreated control group, DMSO: dimethyl sulfoxide, TMZ: temozolomide

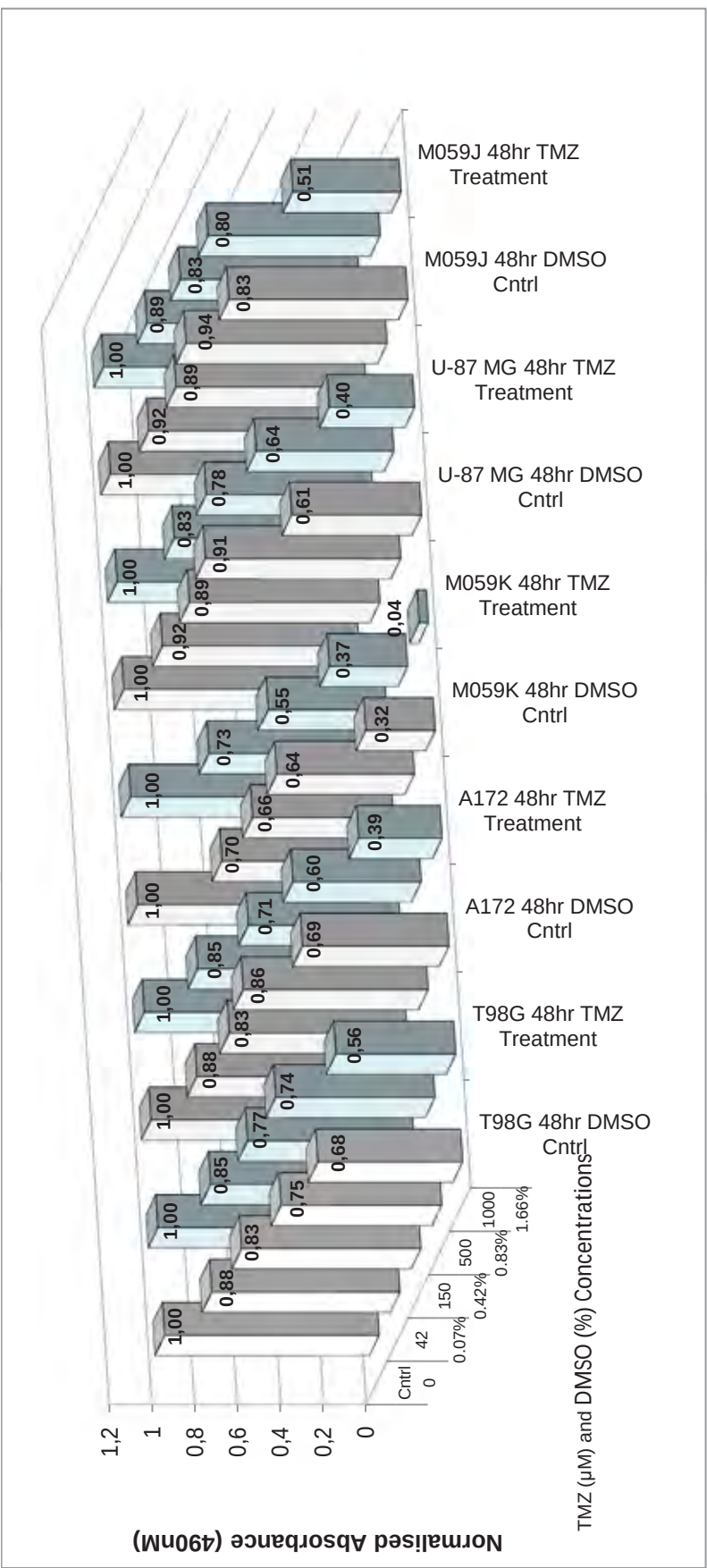


Figure 5.5 Response of glioma cell lines at 48 hr after TMZ treatment (a stock concentration of 60 mM TMZ)

Drug dilutions prepared from a doubly concentrated main stock of TMZ contained half the concentration of DMSO in each dilution of TMZ. Bars represent relative number of total viable cells.

Abbreviations: Cntrl: Untreated control group, DMSO: dimethyl sulfoxide, TMZ: temozolomide

5.3.2 Collection of TMZ IC_{50} Values for the Five Glioma Cell Lines

Final TMZ concentrations of 90 and 120 mM were prepared to determine maximum soluble amount of TMZ in 100% DMSO. While final stock concentration of 90 mM was efficiently dissolved in DMSO, incomplete dissolution of drug particles was observed for drug solution with final TMZ concentration of 120mM. The main stock concentration was then increased to 90 mM from 60 mM in order to reduce DMSO-caused cytotoxicity and collect IC_{50} doses with less interference of DMSO on the individual effect of TMZ. The cells were exposed to the drug dilutions prepared from 90 mM stock solution for 72 and 96 hr.

Figure 5.6 shows TMZ response of five glioma cell lines which included an untreated control and DMSO control groups for each treatment endpoint (72 and 96 hr). DMSO dilutions up to 0.74% was well tolerated by all cell lines except M059K which showed approximately 40% mortality rate when 666 μ M TMZ dilution containing 0.74% DMSO was applied for 96 hr. In U-87 MG cells, treatment with 1.11% DMSO produced a dramatic decrease in cell viability by 30% (see Figure 5.6). Response pattern of the cell lines were slightly improved at 96 hr treatment compared to the dose-response curves obtained at 72 hr treatment. Therefore, IC_{50} doses of the cell lines were collected using 90 mM TMZ stock solution with treatment duration of 96 hr (shown in Figure 5.7). Average dose of 267 μ M TMZ reduced proliferation of M059K cells to 50% in comparison to the proliferation rate of DMSO-only control group. Significantly higher concentrations of TMZ; 843, 860, 681 and 767 μ M were required to reach the IC_{50} in the four glioma cell lines, T98G, A172, U-87 MG, M059J, respectively. Among five glioma cell lines, M059K cells were established to be the most responsive to TMZ compared to the other cell lines examined (see Figure 5.7).

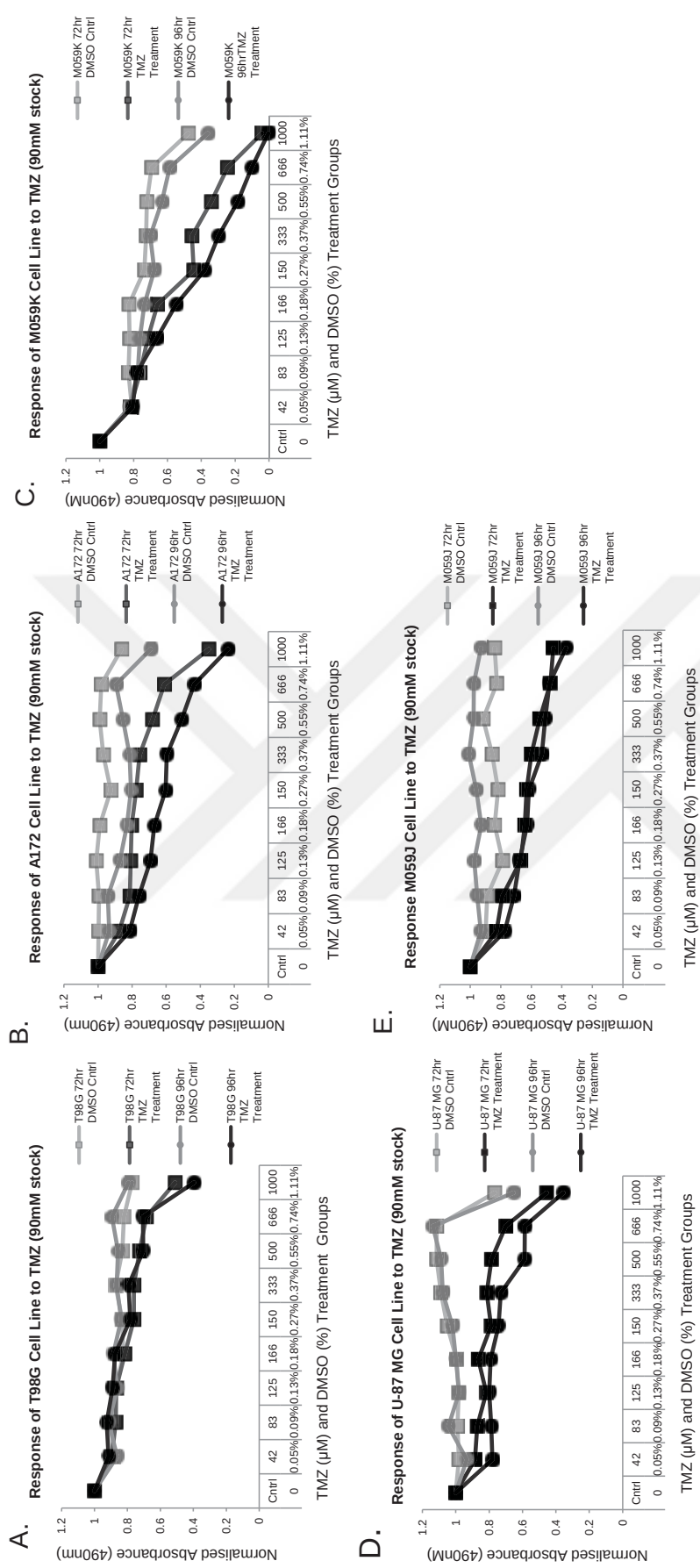


Figure 5.6 Response of glioma cell lines at 72 and 96 hr after TMZ treatment (a stock concentration of 90mM TMZ)

The five glioma cell lines were exposed to a series of TMZ concentrations diluted from 90mM stock solution. TMZ response of the cell lines was measured at two time-points (72 and 96 hr). Black lines represent treatment with TMZ, grey lines represent the DMSO control.

Abbreviations: Ctrl: control group, DMSO: dimethyl sulphoxide, TMZ: temozolomide

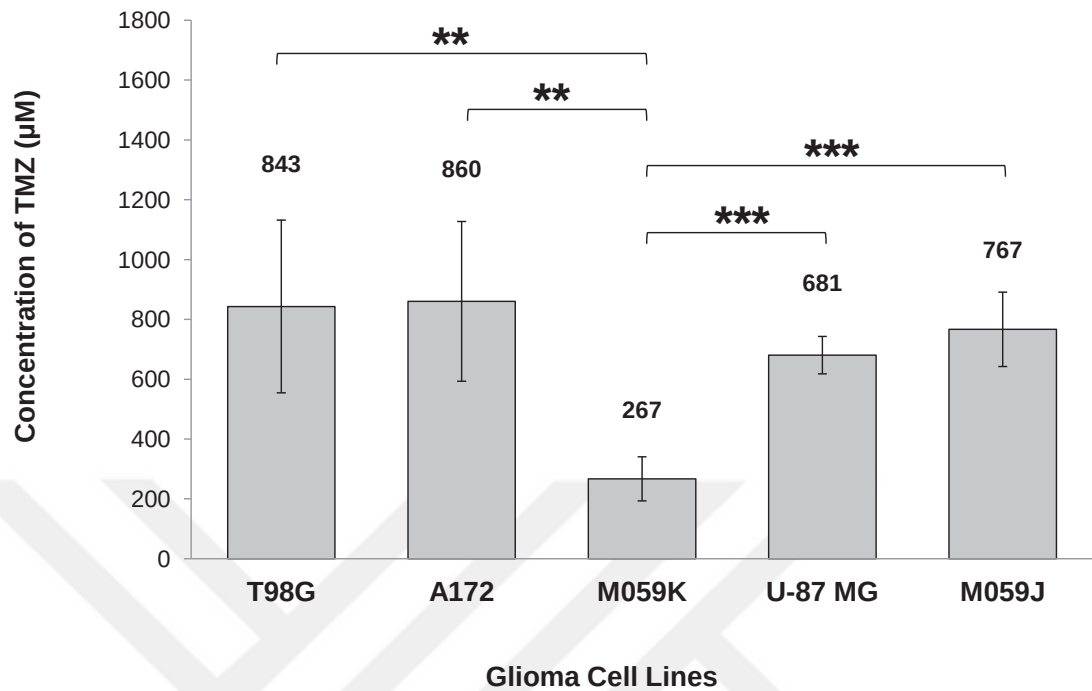


Figure 5.7 IC₅₀ doses (µM) of glioma cell lines at 96 hr post TMZ treatment

Glioma cell lines were treated with a series of temozolomide dilutions (42 to 1000 µM) to determine optimal IC₅₀ concentration for each cell line. Results represent means of triplicate values representative of three separate experiments. Bar represents mean standard deviation. Data was compared using a two-tailed Student's t-test. Asterisks denote significant differences relative to M059K cell line, ** = $p < 0.01$, *** = $p < 0.001$

5.3.3 *MGMT* Promoter Methylation and Protein Expression in the Five Glioma Cell Lines

MGMT promoter methylation was examined in glioma cell lines. CpG genome universally methylated and unmethylated DNAs were used as control samples representing 100% and 0% methylation, respectively. All cell lines were methylated. Interestingly, T98G and M059K cell lines were heterozygous, displaying positive unmethylated bands as well as methylated bands (shown in 5.7, A). Promoter methylation can also be quantified with pyrosequencing. The average methylation percentages at five CpG sites in the *MGMT* promoter region of five glioma cell lines were 45% for T98G, 59% for A172, 50% for M059K, 58% for U-87 MG and 60% for M059J. MSP and pyrosequencing analysis of *MGMT* promoter methylation in five glioma cell lines generated concordant results (see Figure 5.8). Fisher's exact test was used to compare results and no difference ($p = 1.000$) was found between the results obtained by these two methods.

Correlation of percentage *MGMT* promoter methylation with TMZ response was evaluated. Cell lines with *MGMT* promoter methylation ranging from 45% to 60% showed resistance to a wide range of TMZ concentrations tested (up to 1000 μ M), except M059K (50% promoter methylation) which was found to be relatively sensitive to the TMZ treatment. No clear association was established between *MGMT* gene promoter methylation status and TMZ response of the cell lines.

A.

Samples	Methylated Control		Unmethylated Control		T98G		A172		M059K		U-87 MG		M059J	
Primers	M	U	M	U	M	U	M	U	M	U	M	U	M	U
														
Methylation Status	Met		Unmet		Met		Met		Met		Met		Met	

B.

Sample Nr	Met (%)	Met (%)	Met (%)	Met (%)	Met (%)	Mean Met (%)	PSQ Result	MSP Result	MGMT Met	Response Characteristics
UnMeth	0	4	0	0	4	2	U	U	U	
Meth	97	96	93	91	95	94	M	M	M	
T98G	50	49	43	41	41	45	M	M	M	Resistant
A172	87	82	48	43	36	59	M	M	M	Resistant
M059K	49	49	44	42	65	50	M	M	M	Sensitive
U-87 MG	63	70	56	53	46	58	M	M	M	Resistant
M059J	71	77	53	38	63	60	M	M	M	Resistant

Figure 5.8 Promoter methylation status of *MGMT* gene identified using MSP (A) and pyrosequencing (B) methods DNA samples of the five glioma cell lines

The products from control samples were examined both in MSP and pyrosequencing methods. **(A)** The *MGMT* promoter fragments in the control samples were observed at 80 and 92 bp in the lanes which were loaded with the PCR products of the methylated DNA control-methylated primer and unmethylated DNA control-unmethylated primer amplifications, respectively. No bands were observed with the methylated DNA-unmethylated primer and unmethylated DNA-methylated primer control combinations. **(B)** An average percentage of methylation obtained from five CpG islands of *MGMT* promoter showed that all cell lines were methylated with methylation levels greater than 45%.

Note: The percentage of mean *MGMT* promoter methylation value was considered methylated when the percentage was >9%

Abbreviations: **(A)** M: methylated primer, Met: methylated *MGMT* promoter, U: unmethylated primer, Unmet: unmethylated *MGMT* promoter **(B)** M: methylated *MGMT* promoter, Met (%): percentage of *MGMT* promoter methylation, Meth: methylated DNA control, MGMT Met: *MGMT* promoter methylation status, MSP: methylation specific PCR, PSQ: Pyrosequencing, U: unmethylated *MGMT* promoter, Unmeth: unmethylated DNA control

MGMT protein expression levels were measured in the cell lines to determine if we could correlate MGMT protein expression with MGMT promoter methylation status and TMZ response. MGMT protein was detected in two of the five glioma cell lines (see Figure 5.9). MGMT protein was absent in U-87 MG and M059J cells. In addition, low levels of protein were found to be expressed in A172 cells. As expected, loss of MGMT protein expression was concordant with methylated MGMT promoter, however high protein expression levels of MGMT were observed in T98G and M059K cell lines. Moreover, no direct association was established between MGMT protein expression levels and TMZ response of the cell lines. Neither lack of expression of the MGMT protein, nor highly methylated *MGMT* promoter detected in the cell lines was found to be related to the responsiveness to TMZ the cell lines.

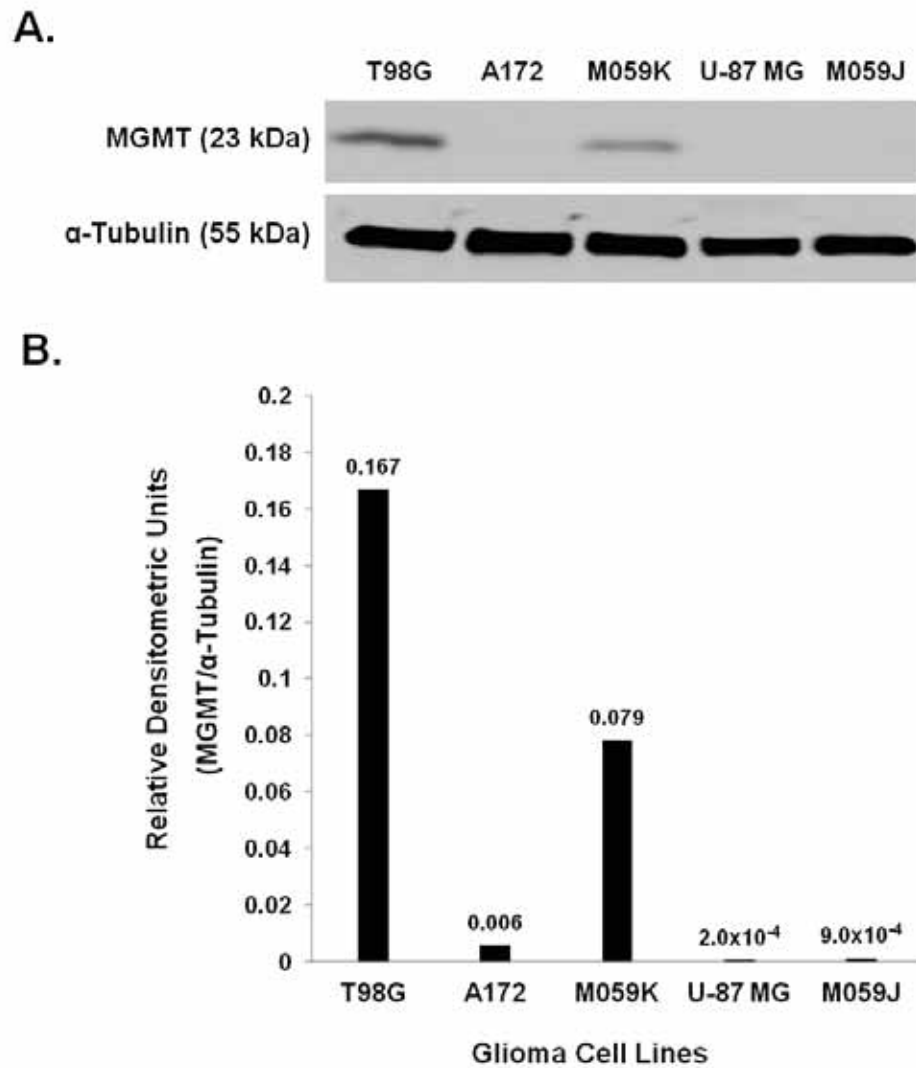


Figure 5.9 MGMT protein expression levels in the five glioma cell lines

Representative immunoblotting **(A)** Quantitative immunoblot analysis **(B)** MGMT protein expression levels were assessed by immunoblotting. MGMT protein expression was detected using specific mouse monoclonal antibody, clone MT3.1. The experiment was repeated three times with similar results, and one representative immunoblot is shown.

5.3.4 *MGMT* Promoter Methylation and Protein Expression in a cohort of 42 glioblastoma samples

Analyses of *MGMT* promoter methylation in 42 glioma samples were performed using both MSP and pyrosequencing methods. *MGMT* promoter methylation was successfully detected in 36/42 glioma patients (see Figure 5.10 and Table 5.12). Six samples failed which could possibly be due to low DNA yield extracted from FFPE tissue. A methylation rate of 26% (n = 11) was detected in this glioma cohort (n = 42).

Methylation rate in dichotomised samples according to their classification as responders or non-responders to standard therapy was separately evaluated. Table 5.13 shows the distribution of the *MGMT* promoter methylation status in response cohort. No difference was detected between the percentages of the samples with methylated *MGMT* promoter in responders and non-responders. The *MGMT* promoter was found to be methylated in 33% of the response cohort including 27 samples.

MGMT protein expression was also examined in the tumour samples (shown in Figure 5.11). T98G cell extracts were used as positive control for *MGMT* protein expression. Differential protein expression profiles were observed for all 42 samples. Detectable levels of *MGMT* protein expression was observed in 14 tumour samples (sample number; 2, 4, 7, 9, 11, 13, 14, 15, 21, 26, 27, 30, 32, 34). 14 tumour samples expressing *MGMT* protein included three samples (21.4%) with methylated, 10 samples (71.42%) with unmethylated *MGMT* promoter and one sample with unknown methylation status. Unexpectedly, protein expression was identified in the samples with methylated *MGMT* promoter. In addition, a number of samples with unmethylated *MGMT* promoter (for example; sample number 18, 23, 24, 25, 36, 40, 41, 42) showed no detectable protein

expression. Protein levels did not completely represent *MGMT* promoter methylation status of the samples.

Spearman correlation coefficients were computed and relationship between *MGMT* promoter methylation status (methylated / unmethylated) and protein expression (presence / absence) and their correlation with response (responders / non-responders) were assessed. There was no significant ($p > 0.05$) correlation between *MGMT* promoter methylation status and protein expression when associated with response (see Table 5.14).

Overall survival (OS) in relation to *MGMT* promoter methylation status and protein expression was evaluated by Kaplan-Meier survival curves (see Figure 5.12). No significant difference was observed between the OS of patients with and without methylated *MGMT* promoters ($p = 0.388$, Log Rank test). Median survival was 18.3 and 17.3 months for the patients had methylated and unmethylated promoter, respectively. *MGMT* protein expression did not significantly impact on survival either ($p = 0.910$, Log Rank test). Estimated median survival was 17.9 months in the group of patients with an absence of *MGMT* expression while the analysis of the patient samples expressing *MGMT* protein showed a median survival of 18.3 months.

Table 5.12 MGMT promoter methylation status analysed using pyrosequencing method in 42 glioma samples

Sample ID	Met (%)	Met (%)	Met (%)	Met (%)	Met (%)	Mean Met %	PSQ Result	MSP Result	MGMT Met	PFS6	OS
UnMeth	3.58	0	0	3.14	3.71	2.1	U	U	U		
Meth	97.02	96.89	96.55	92.3	94.15	95.4	M	M	M		
1	2.29	2.91	5.03	2.72	4.3	3.5	U	X	U	R	21.20
2	2.08	4.52	3.88	1.99	4.85	3.5	U	U	U		9.97
3	49.79	35.24	16.34	46.57	49.16	39.4	M	X	M		24.4
4	15.21	14.84	13.69	8.81	18.46	14.2	M	M	M	NR	17.40
5							X	M	M	R	20.83
6	3.57	3.15	3.99	2.51	3.49	3.3	U	X	U		21.60
7	2.89	2.44	4.7	7.96	2.89	4.2	U	X	U	R	12.90
8							X	X	X		
9	2.32	2.45	2.78	1.75	2.86	2.4	U	X	U		10.53
10	15.06	10.95	10.01	13.67	5.84	11.1	M	U	M	NR	24
11							X	U	U		17.03
12							X	X	X		
13	20.04	0	23.3	18.44	0	12.4	M	X	M	R	14.30
14	7.47	9.17	9.77	0	9.74	7.2	U	X	U		22.67
15							X	X	X	NR	2.50
16	24.92	61.36	64.58	58.43	16.69	45.2	M	X	M	R	21.40
17							X	X	X	NR	3.63
18	0	10.55	0	26.01	5.64	8.4	U	X	U		10.83
19							X	U	U	NR	20.07
20	21.31	18.8	11.08	11.61	4.44	13.5	M	U	M		13.93
21							X	U	U	R	30.23
22							X	X	X		9.87
23							X	U	U		8.87
24	5.98	1.78	6.23	2.3	3.32	3.9	U	M	U	R	15.17
25	5.18	0	7.03	5.26	3.56	4.2	U	U	U	R	14.00
26	3.22	5.98	3.36	4.9	0	3.5	U	M	U	NR	11.73
27	6.71	3.14	6.05	5.15	3.84	5.0	U	U	U	R	50.30
28	2.14	2.3	3.65	0	3.29	2.3	U	U	U	NR	13.37
29	27.86	8.52	9.88	10.11	22.34	15.7	M	X	M	R	22.40
30	4.34	2.34	4.35	2.67	2.24	3.2	U	X	U	NR	9.50
31	0	2.68	2.8	0	5.62	2.2	U	M	U		13.73
32	6.9	9.72	7.79	6.2	11.99	8.5	U	U	U	R	29.90
33	12.22	6.77	8.09	6.26	5.23	7.7	U	X	U	R	19.93
34	20.68	20.87	20.53	18.41	12.6	18.6	M	X	M	NR	10.77
35	3.92	2.61	5.78	2.29	1.79	3.3	U	M	U		7.20
36	3.07	2.52	2.4	2.41	2.5	2.6	U	X	U	R	10.63
37							X	X	X	NR	8.07
38	0	8.44	23.07	18.37	17.25	13.4	M	X	M	R	11.47
39	26.72	32.85	28.52	29.39	14.2	26.3	M	X	M	NR	20.80
40							X	U	U	R	31.17
41	2.08	4.52	3.88	1.99	4.85	3.5	U	X	U	NR	9.23
42	7.65	0	2.92	2.12	2.23	3.0	U	X	U		12.50

MGMT promoter methylation was identified in 36 of a total of 42 patients

Abbreviations: LTS: long-term survivors, M: methylated MGMT promoter, Met (%): percentage of MGMT promoter methylation, Meth: methylated DNA control, MSP: methylation specific PCR, NR: non-responder, OS: overall survival (months), PFS6: six-month progression free survival, PSQ: Pyrosequencing, R: responder, Unmeth: unmethylated DNA control, U: unmethylated MGMT promoter, X: unidentified MGMT promoter methylation status.

Note: the percentage of mean MGMT methylation value was considered methylated when the percentage was > 9%. Letters highlighted red show discrepancy of results from MSP and pyrosequencing.

Table 5.13 Summary of *MGMT* promoter methylation of tumour samples in response cohort

			Methylated MGMT Promoter	Unmethylated MGMT Promoter	Unidentified Samples
Response Cohort (n=27)	Non-responders	n	4	5	3
		%	33.33	41.66	25
	Responders	n	5	10	0
		%	33.33	66.66	0
	Total	n	9	15	3
		%	33.33	55.55	11.11

Abbreviations: %: percentage of patients with methylated, unmethylated MGMT promoter and unidentified samples, n: number of samples

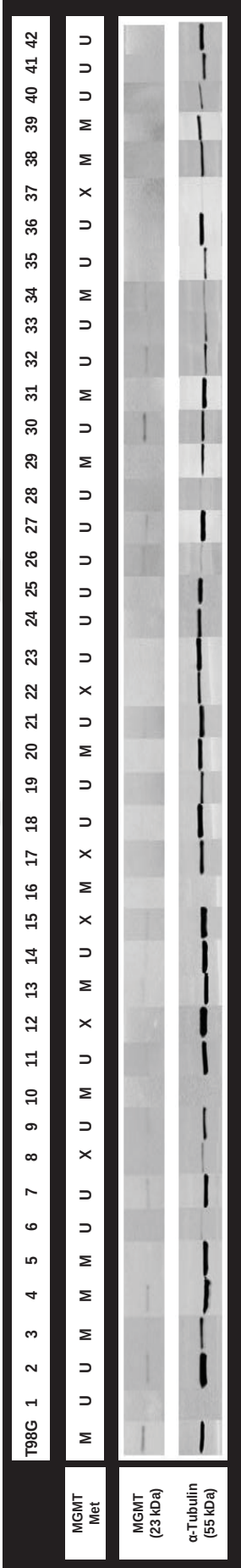


Figure 5.11 MGMT protein expression in 42 tumour samples

Although the α -tubulin levels were appeared to be unevenly distributed, total protein levels were measured by total protein assay to load equal amount of protein (30 μ g/sample/well) into each well.

Abbreviations: M: methylated MGMT promoter, MGMT Met: methylation status of MGMT promoter, U: unmethylated MGMT promoter, X: unidentified MGMT promoter methylation status

Note: Protein extracts from T98G cell lines was used as positive control for MGMT protein expression.

Table 5.14 Spearman correlation coefficients calculated to demonstrate the relationship between response characteristics of the patients and *MGMT* promoter methylation and *MGMT* protein expression detected in the patient tumour samples.

			Response Cohort Responders / Non-responders	<i>MGMT</i> Promoter Methylation Status Methylated / Non-methylated	<i>MGMT</i> Protein Expression Presence / Absence
Spearman's rho	Response Cohort Responders / Non-responders	Correlation Coefficient	1.000	-0.111	-0.111
		Sig. (2-tailed)		0.605	0.605
		N	24	24	24
	<i>MGMT</i> Promoter Methylation Status Methylated / Non-methylated	Correlation Coefficient	-0.111	1.000	-0.67
		Sig. (2-tailed)	0.605		0.757
		N	24	24	24
	<i>MGMT</i> Protein Expression Presence / Absence	Correlation Coefficient	-0.111	-0.067	1.000
		Sig. (2-tailed)	0.605	0.757	
		N	24	24	24

Abbreviations: N: number of samples

Note: Three patients were omitted due to the lack of determined *MGMT* promoter methylation status

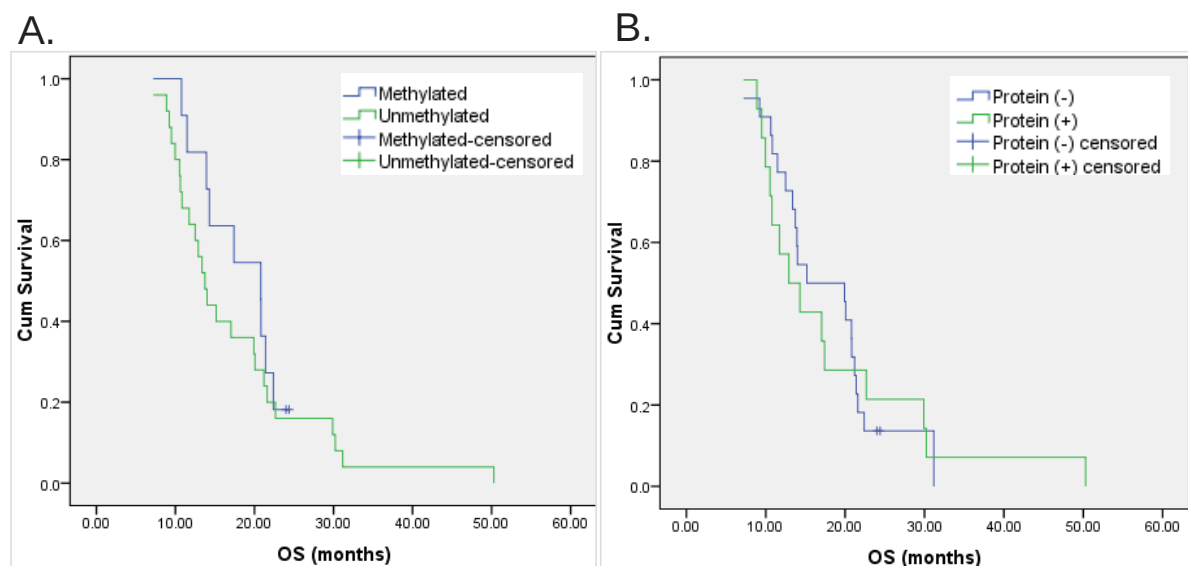


Figure 5.12 Kaplan Meier analysis comparing overall survival of patients with and without methylated *MGMT* promoter (A) and *MGMT* protein expression (B)

5.4 DISCUSSION

The key objective of this study was to investigate the correlation of *MGMT* promoter methylation status and *MGMT* protein expression levels with TMZ response in five glioma cell lines and with response to standard therapy including TMZ treatment in 42 glioblastoma tumour samples. The five glioma cell lines were exposed to a wide range of TMZ dilutions (42 to 1000 μ M) to determine response to the drug. Individual IC_{50} values of TMZ were obtained for the cell lines using MTS cell proliferation assay. Among the five cell lines used in this study, M059K cell line was found to be the most sensitive to TMZ treatment. Variable responses for temozolomide have been reported by other studies. In a study using glioma cell lines treated with TMZ for 72 hr, IC_{50} s for A172 and U-87 MG were less than 200 μ M, while IC_{50} of TMZ could not be reached for T98G at the highest dose (1000 μ M) (Kanzawa et al., 2004). Yoshino and colleagues have reported TMZ IC_{50} values of 441.6 and 52.4 and 23 μ M for T98G, A172 and U-87 MG cell lines, respectively (Yoshino et al., 2010). Inconsistent results from different studies can also be explained by the fact that majority of the commercial cell lines derived from primary gliomas undergo innumerable changes due to continuous passaging in vitro and possibly accumulate diverse chromosomal and gene mutations in addition to the known genetic alterations (Calogero et al., 2011; Masters, 2000). The commercial glioma cell lines used in this study was, unfortunately, poorly representative of primary glioblastoma models. For example, all of the cell lines were methylated at the *MGMT* promoter which is not reflective of the incidence of *MGMT* promoter methylation in the patient population (~35%)

MGMT promoter methylation was determined in all of the glioma cell lines using both MSP and pyrosequencing methods. Methylated *MGMT* promoter (ranging from 45 to 60% with pyrosequencing) was detected in all glioma cell lines examined. Both

methods were concordant with the outcome. However, conclusions cannot be made since all cell lines were methylated and no TMZ effects were detected. Using methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) and quantitative MSP methods, *MGMT* promoter methylation in 11 glioma cell lines was not found to be correlated with TMZ response (van Niftrik et al., 2010).

In tumour samples, *MGMT* promoter methylation status was identified in 36 (86%) out of 42 using both MSP and pyrosequencing methods. 6 cases (50%) showed discordant results. MSP only provides information about presence or absence of the methylation and interrogates 3-5 CpGs, whereas, quantitative results can be obtained by pyrosequencing which examines up to 12 CpG sites (Weller et al., 2010). Even though no agreement exists regarding the use of methods to analyse *MGMT* promoter methylation, pyrosequencing technique offers an improved prediction of overall survival compared to MSP and SQ-MSP (Karayan-Tapon et al., 2010). Various studies have reported that patients with methylated *MGMT* promoter form 30.4 to 54.8% of the entire primary glioblastoma cohorts (Brell et al., 2005; Esteller et al., 2000; Hegi et al., 2005; Nakamura et al., 2001; Paz et al., 2004). A methylation rate of 26% (n = 11) found in our glioma cohort was in good agreement with previous reports (Nakamura et al., 2001; Paz et al., 2004).

The incidence of *MGMT* promoter methylation in our small patient cohort was 26%. We found no difference in our patient cohort in regard to survival stratified according to *MGMT* promoter methylation. Additionally, there was no difference in response to treatment measured by PFS6.

Our cohort was too small to confirm that of several other reports investigating the prognostic power of *MGMT* promoter methylation which have indicated a survival

advantage in the patient groups with methylated *MGMT* promoter (Hegi et al., 2005; Stupp et al., 2009; van den Bent et al., 2009a; Wick et al., 2009). Two different studies conducted in patients with anaplastic glioma and oligodendrogliomas suggested that *MGMT* promoter methylation status alone is not a potent predictive factor for the response to treatment with alkylating agents (van den Bent et al., 2009a; Wick et al., 2009).

MGMT protein expression levels were assessed in the five glioma cell lines and 42 tumour tissue samples by immunoblotting. Variable *MGMT* protein expressions were observed in the tumour samples as well as the cell lines. van Nifterik and colleagues suggested that *MGMT* protein expression detected by immunoblotting in 11 glioma cell lines was predictive of response to TMZ with 100% certainty. The cell lines with detectable *MGMT* protein expression were reported to be sensitive, while loss of protein expression resulted in sensitivity to TMZ (van Nifterik et al., 2010). In contrast, whilst A172, U87MG and M059J all showed absent *MGMT* protein expression, *MGMT* expression was clearly present in extracts from T98G and M059K. The lack of correlation observed between the *MGMT* promoter methylation status of the cell lines and TMZ response is again most likely due to the use of commercial cell lines exhibiting critical non-responsiveness to chemotherapeutic agents. Mellai *et al.* indicated a significant correlation between immunoblotting and IHC results, however, no association of IHC results with *MGMT* promoter methylation was reported (Mellai et al., 2009). The reports concerning the lack of association between methylation status of *MGMT* promoter and protein expression have been previously published by several research groups using immunohistochemistry (Brell et al., 2005; Jeuken et al., 2007; Preusser et al., 2008; Rodriguez et al., 2008).

To conclude, we showed that *MGMT* promoter methylation could be detected with both pyrosequencing and MSP. All cell lines and 26% of the tumours were methylated. The correlation between *MGMT* promoter methylation in tumour samples and overall survival was investigated. Discordance between *MGMT* promoter methylation and response to TMZ treatment; and *MGMT* promoter methylation and protein expression require further investigation in a larger cohort.



CHAPTER 6

PROTEOMIC DISCOVERY OF BIOMARKERS

ASSOCIATED WITH CHEMOTHERAPY

RESPONSE IN GLIOMA USING SELDI-TOF MS

6. 1 INTRODUCTION

Proteins reflect genetic program of the cells and interconnect this genomic data with cellular behaviour through complex biological pathways (Dove, 1999). Utility of proteomics has been demonstrated in different areas of clinical medicine; Alzheimer's disease (Butterfield et al., 2003), cardiovascular (Balestrieri et al., 2008) and autoimmune diseases (Hueber and Robinson, 2006). There is a growing interest in the application of proteomics technologies in cancer research to discover biomarkers for early cancer detection and diagnosis, therapy efficacy and also to investigate protein-drug interactions (Hanash, 2003; Tyers and Mann, 2003). Discovery and development of biomarkers which can be detected through simple, non-invasive or minimally invasive methods is an important goal for oncology. Robust and efficient biomarker identification methods which can be implemented to identify cancer risk, improve early diagnostic and facilitate accurate grading and treatment monitoring are priority (Somasundaram et al., 2009). Standard treatment of glioblastoma includes total resection of the tumour, radiotherapy with concurrent and adjuvant TMZ. However, not

all patients (response rate: 11 to 47%) with gliomas respond to therapy regimen with TMZ which was linked to a chemoresistance mechanism caused by an unmethylated MGMT promoter (Bower et al., 1997; Brandes et al., 2001; Friedman et al., 1998; Yung et al., 1999). As discussed earlier in Section 1.1.5.3, Literature Review-Chapter 1, methylation status of MGMT promoter is the only predictive factor which was suggested to be a potential marker for response to standard treatment with TMZ in glioblastoma patients (Hegi et al., 2005; Stupp et al., 2009). As opposed to this, NOA-04 phase III trial indicated that promoter methylation of MGMT alone is not sufficient to determine response outcome of patients with grade III, anaplastic glioma (Wick et al., 2009). The most remarkable finding of this study was the significantly different PFS which was observed between patients with and without promoter methylation of MGMT in the patient group received radiotherapy alone. Therefore, methylated MGMT promoter is proposed to be utilised in glioma treatment as an indicative of better survival outcome and possibly a predictive marker for response to radiotherapy itself, independent of TMZ. However, inconsistencies exist in the literature regarding its ability of correctly predicting patient response (Hegi et al., 2005; Stupp et al., 2009; Wick et al., 2009). Our data presented in Chapter 5 showed that there was no concordance between MGMT promoter methylation status and response to TMZ treatment in glioma patients. Due to complexity of response mechanisms interfering with chemotherapy resistance in glioblastoma, new strategies for the discovery of biomarkers with utility are demanded. The different proteomic techniques have been discussed in detail in Section 1.4.2, Literature Review-Chapter 1. SELDI-TOF MS was chosen for the current research because it is an effective array technology to analyse complex biological materials and to profile peptides for biomarker identification (Seibert et al., 2004). Applications of SELDI-TOF MS technology in biomarker

discovery of a number of cancer types and applications of ProteinChip SELDI-TOF MS technology in glioma and other brain tumours were discussed extensively in Literature Review, Chapter 1 and summarised in Table 6.1 and 6.2, respectively.



Table 6.1 Summary of publications on a number of cancer types using SELDI-TOF MS

Cancer Type	Sample	Function	Identified Biomarkers	References
Ovarian	Serum	Detection of early stage ovarian cancer	Apo AI, TT, ITIH4	(Zhang et al., 2004)
	Serum	Discrimination of benign and malignant	Apo AI, TT, CTAPIII combined with CA125	(Clarke et al., 2011)
Breast	Serum	Predictive for treatment with docetaxel	Kininogen, Apo AII	(Heike et al., 2005)
Prostate	Serum	Discrimination of benign and malignant	An algorithm consisting seven proteins	(Petricoin et al., 2002b)
Colorectal	Serum	Discrimination of healthy from cancer samples	Four-proteins with m/z 5911, 8930, 8817 and 4476	(Chen et al., 2004)
	Serum	Predictive for treatment with oxaliplatin and capecitabine	6 proteins at m/z 3330, 2756, 6847, 3893, 3978 and 2055	(Helgason et al., 2010)
Pancreatic	Serum	Prognostic	Apo CI and a truncated form of Apo CI	(Takano et al., 2008)

Abbreviations: Apo AI: apolipoprotein AI, Apo AII: apolipoprotein AII, Apo CI: Apolipoprotein CI, CTAPIII: Connective Tissue Activating Protein III, ITIH4: cleavage fragment of inter-alpha-trypsin inhibitor heavy chain H4, TT: truncated form of transthyretin

Table 6.2 Summary of publications on glioma proteomics using SELDI-TOF MS

Research Group	Source	Groups in comparison	Identified Biomarkers
Hobbs <i>et al.</i> 2003	Tissue (n = 4)	- CE vs. NE regions from glioma patients	-Distinct protein profiles between CE and NE regions
Liu <i>et al.</i> 2005	Serum (n = 105)	-Low grade astrocytoma (Grades I,II) vs. high grade astrocytoma (AA, glioma) -Astrocytoma vs. benign brain tumours -Cancer vs. healthy controls	-Three peaks -20 peaks -15 peaks significantly differentially expressed
Khwaja <i>et al.</i> 2006	CSF (n = 32)	-Neoplasms including five gliomas, CNS inflammatory disease and control samples	-Overexpression of Carbonic anhydrase detected in neoplasms and identified as a prognostic marker
Wibom <i>et al.</i> 2006	Animal model (n = 24)	-Radiation treated vs. untreated	-Detected 77 differentially expresses peaks
Bouamrani <i>et al.</i> 2006	Tissue (n = 10)	Gliomas vs. oligodendrogliomas	Identified three potential biomarkers
Zhang <i>et al.</i> 2007	Serum (n = 140)	Astrocytoma vs. healthy controls Astrocytoma Grade I and II vs. AA and Glioma	-Revealed a panel of seven protein peaks -Two protein peaks
Petrik <i>et al.</i> 2008	Serum (n = 200)	Healthy Astrocytoma Grade II AA Glioma	-AHSG identified as a downregulated biomarker with increasing astrocytoma grade.
Wibom <i>et al.</i> 2010	Animal model (n = 27)	Vandetenib (anti-angiogenesis treatment) treated vs. control animals	Identified a significantly different protein expression pattern in glioma and normal brain samples following vandetenib treatment

Abbreviations: AA: anaplastic astrocytoma, AHSG: B-chain of α_2 -Heremans-Schmid glycoprotein, CE: contrast enhanced, CNS: central nervous system, CSF: cerebrospinal fluid, NE: non-enhanced

Working Hypothesis of this chapter:

Analysis of SELDI-TOF MS protein profiles reveal differentially expressed proteins between responder and non-responder patient groups and also between long-term survivor (LTS) and short-term survivor (STS) groups to be used as potential predictive and/or prognostic biomarkers.

The main objective of this study was to discover potential biomarkers to differentiate responders from non-responders and LTS group from STS.

The specific aims of this chapter were to:

1. Analyse fresh-frozen tumour samples (n = 42, training cohort; Training cohort included 42 glioblastoma patients whose fresh-frozen tumour samples were used in SELDI-TOF MS study for the identification of predictive and prognostic biomarkers) using SELDI-TOF MS and detect differentially expressed proteins between tumour samples of responders and non-responders and also LTS and STS.
2. Generate a panel of biomarkers with high sensitivity and specificity using Binary Logistic Regression (BLR) analysis and determine predictability of these biomarkers individually and in combination as accurate predictors of patient response to standard therapy or prognostic indicators of overall survival.
3. Identify names of candidate biomarkers with powerful discriminative ability and target these proteins using specific antibodies by performing immunoblotting in training cohort. Present preliminary results from the study conducted to validate biomarkers using immunohistochemistry in an independent cohort of 66 patients (validation cohort).

6.2 METHODS

Discovery and validation processes of biomarkers consist of various technologies with multiple steps of analyses (see Figure 6.1). Global protein expression profiling of glioma cell lines and tumour specimens were performed using protocols defined in the following sections.

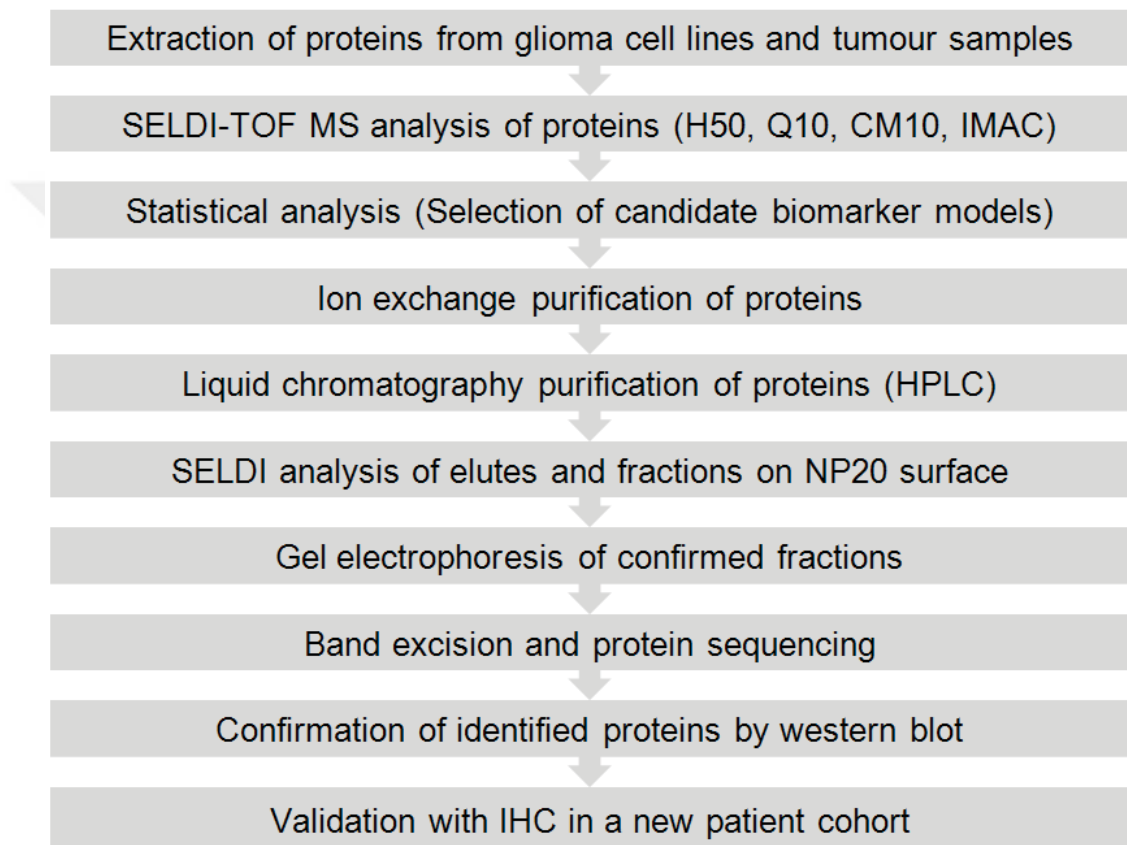


Figure 6.1 The workflow followed for biomarker discovery

Abbreviations: CM10: cation exchange, H50: Hydrophobic, HPLC: High Performance Liquid Chromatography, IMAC: immobilised metal affinity capture, Q10: anion exchange

6.2.1 Protein Extraction Protocols

6.2.1.1 Protein Extraction from Cell Lines for SELDI-TOF MS

1.5×10^6 cells from four cell lines (T98G, A172, M059K, U-87 MG) were seeded in T25 flasks (Media components for cell lines can be found in section 2.2.1). The flasks were incubated for 24 hr in a humidified atmosphere with 5% CO₂ at 37⁰C. After 24 hr incubation, media was removed and cells were washed 3 times with ice-cold PBS. Protein lysis buffer (150 µl) (see Table 6.3) was directly added on cells and cells were scraped with cell scraper. Cell suspension was collected and transferred into a 15ml centrifuge tube. Further homogenisation was continued by sonication using the vibra cell sonicator (John Morris Scientific, NSW, Australia) for 20 seconds followed by 2 minutes of cooling and repeated 6 times to total sonication time of 2 minutes. The samples were transferred into QiaShredder spin columns (Qiagen, Hilden, Germany) and centrifuged at 13,500 x g for 5 minutes to remove any insoluble debris. The lysates were then aliquoted into 20 µl aliquots and stored at -80 ⁰C until analysis.

Table 6.3 Protein lysis buffer components for SELDI-TOF MS analysis

Components	Amounts (g-ml)	Final Concentrations (%-M)
Urea	30 g	9.5 M
CHAPS	1 g	2%
DTT*	0.5 g	1% (64.8 mM)
MilliQ water	50 ml	

*DTT was prepared and added separately due to its potential interference with protein quantification methods.

Note: Optimisation of protein lysis buffer was described in Section 6.3.1.

6.2.1.2 Protein Extraction from Tumour Samples for SELDI-TOF MS

Microcentrifuge tubes (1.5 ml) were weighed and rapidly cooled on dry ice. Balance weight was set to zero to exclude the weight of the tube alone to allow estimation of the tumour sample weight. The fresh frozen glioma tumour tissue samples ~50 mg were ground to a fine powder using a dry-ice cooled mortar and pestle in approximately 10ml of liquid nitrogen. Sample powder was collected from the mortar and transferred into the microcentrifuge tube. Then the tube containing the macerated tumour tissue was re-weighted to obtain the weight of the tissue. Lysis buffer (10 µl; 9.5 M urea, 2% CHAPS in milliQ water) (see Table 6.3) was added to 1mg of tumour sample and mixed thoroughly by pipetting up and down and then incubated for 30 minutes on ice. Following steps were conducted in a similar manner as described for tumour tissue samples. 2 minutes of fractionated sonication was performed for the homogenisation of cells and the lysates were centrifuged using QiaShredder (Qiagen, Hilden, Germany) at 13,500 x g for 5 minutes. Filtrate was dispensed into microcentrifuge tubes as 20µl aliquots and stored at -80 °C until analysis.

6.2.2 Protein Quantification Method using DC™ Protein Assay

Proteins from cell extracts were tested to determine protein concentration in the presence and absence of DTT. DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) was performed as described in Section 2.5.2, Materials and Methods, Chapter 2. Dilutions of BSA protein standards (Genesearch Pty. Ltd., Aundel, QL, Australia; 0.132 mg/ml, 0.197 mg/ml, 0.296 mg/ml, 0.44 mg/ml, 0.66 mg/ml and 1mg/ml) were prepared in lysis buffer with and without DTT. Reagent A+S was prepared by addition of 20 µl of Reagent S for each ml of Reagent A in a centrifuge tube. BSA standards (5 µl) and protein samples (1/50 dilution) were transferred into a

clean microtiter plate in duplicate. Reagent A+S (25 µl) and Reagent B (200 µl) were then added into each well. Blank wells containing only the reagent mixture were included in the experimental design. The microplate was vigorously shaken for 5 seconds on a TecanTM Sunrise® Special Plate Reader using Magellan3TM software then incubated for 15 minutes. Following termination of colour development, absorbance of the solution was measured at 650nm.

6.2.3 Preparation of ProteinChip Arrays for Analysis using SELDI-TOF MS

Identifying biomarkers using ProteinChip technology combined with SELDI-TOF-MS involved a number of steps such as sample preparation, protein chip buffer pH selection and application, optimisation of laser intensities, loading samples on chip, removal of unbound proteins, SELDI-TOF MS parameter settings, data pre-processing and cluster formations of the data. Four different ProteinChip® array surfaces; hydrophobic (H50), anion exchange (Q10), cation exchange (CM10), immobilised metal affinity capture (IMAC) (Bio-Rad, Hercules, CA, USA) were used to analyse extracted protein samples. ProteinChip arrays are a 6.8 cm in length with eight 2 mm chemically modified spots on the chip surface. ProteinChip arrays and binding buffers were prepared according to the manufacturer's instructions (Bio-Rad Laboratories, CA, USA). Samples were applied on each surface type in duplicate with a total 10µg protein per spot by diluting in binding buffers recommended and optimised for ProteinChip arrays. DTT was separately added to the sample mixture prior to analysis.

6.2.3.1 Sample Preparation for H50 Array

H50 arrays were pre-equilibrated with 5 µl of 50% acetonitrile for 5 minutes at RT in a humidified chamber. 50% Acetonitrile buffer was carefully removed and spots were then washed with milliQ water. These steps were repeated twice. The array was allowed to dry in order to prevent cross-contamination between spots. Following equilibration of

the array, binding buffer (5 μ l; 10% Acetonitrile, 0.1% TFA) (see Appendix B) was applied two times for 2 minutes each time to pre-wet spots. Binding buffer was removed thoroughly and replaced with 5 μ l of sample. Arrays were then incubated for 2 hours in a humidified chamber on Multi function 3D rotator (Grant Instruments, Chambridgeshire, UK). After incubation, each spot was washed three times with binding buffer for 2 minutes to remove unbound proteins and rinsed twice with MilliQ water and allowed to dry for 15 minutes at RT. Energy absorbing molecule (1 μ l), sinapinic acid (EAM) (see Appendix B) was dispensed to the array spots and let dry. This process was repeated one more time and maximum number of 12 arrays were loaded on the ProteinChip cassette and then analysed by SELDI-TOF MS (PCS 4000 Enterprise Edition, Bio-Rad Laboratories, Fremont, CA, USA).

6.2.3.2 Sample Preparation for Q10 Array

Q10 arrays were treated with binding buffer (5 μ l; 50 mM Tris-HCl, pH 4.0) (see Appendix B) for 5 minutes, twice. Samples (5 μ l) were applied and incubated in a humidified chamber on rotator for 2 hours. Incubation was followed by the removal of excess protein sample by washing three times with binding buffer (5 μ l) for 2 minutes on a rotator. Each spot was rinsed with milliQ water and air-dried for 15 minutes at RT. Application of EAM and analysis of samples were performed in a similar manner as explained in Section 6.2.3.1.

6.2.3.3 Sample Preparation for CM10 Array

CM10 arrays were pre-equilibrated with binding buffer (5 μ l; 50 mM Sodium Acetate, pH 4.0) (see Appendix B) twice for 5 minutes. The binding buffer was removed and the spots were incubated with protein samples (5 μ l) for 2 hours in a humidified chamber on rotator. After incubation, spots were washed three times with binding buffer followed

by a final rinse of the spots twice with MilliQ water and air-drying for 15 minutes. EAM was applied on spots and arrays were prepared for analysis as described in Section 6.2.3.1.

6.2.3.4 Sample Preparation for IMAC Array

For the pre-treatment of IMAC arrays, 0.1 M copper sulphate (5 µl) (Sigma-Aldrich, St Louis, MO, USA) (see Appendix B) was applied on spots for 10 minutes on rotator. Copper sulphate solution was replaced with MilliQ water (5 µl) and spots were washed for 1 minute. Spots were treated with Neutralisation buffer (5 µl; 0.1 M sodium acetate, pH 4.0) (see Appendix B) and incubated for 5 minutes and then quickly rinsed with milliQ water for 1 minute. Binding buffer (5 µl; 1X PBS buffer, pH 7.2; Amresco, Solon, OH, USA) was applied on each spot for 5 minutes on rotator and this process was repeated twice. Binding buffer was aspirated and spots were treated with samples (5 µl) for 2 hours in a humidified chamber on rotator. Following incubation, each spot was washed three times with binding buffer (5 µl) for 5 minutes on rotator and followed by two quick rinses with MilliQ water (5 µl). Arrays were then allowed to air-dry for 15 minutes. Final treatments of spots with EAM and data collection were performed as explained for H50 arrays in Section 6.2.3.1.

6.2.4 Laser Intensities and Binding Buffers

Spot protocols were optimised using protein extracts of cell lines on each different ProteinChip arrays. Laser energies of SELDI laser settings are defined in nanojoules (nJ). Different laser intensities; 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800 and 3000 nJ were tested to decide optimal energy imparted on targets on array surfaces and to create an optimised protocol, for the collection of spectra. Selection of the proper laser intensities was verified by visual evaluation of spectrum. 530 laser shots were

acquired for each spectrum following two warming shots which was not included in the spectrum.

Optimisation of binding buffers for Q10 and CM10 were conducted with the use of buffers with different pHs prior to analysis of full data. 50mM Tris-HCl buffers with pH 7.5, pH 8.0 and pH 8.5 were analysed to determine the optimal pH conditions for Q10 array. Buffers consisting of 50mM sodium acetate and 50 mM ammonium acetate with varying pH values (4.0, 5.0 and 6.0) were applied on CM10 spots and the collected spectra were evaluated visually to select the most efficient buffer and pH conditions.

6.2.5 Classification of Samples

Details of human research ethics approval and the clinical data used in this study were described in detail in Section 5.2.1 and 5.2.2, Chapter 5. For the analysis of response outcome two endpoints were defined. Primary endpoint was 6 months of progression free survival (PFS6) and defined as the time from treatment initiation until tumour progression. Tumour progression was defined by clinician recommendation (based upon scan, symptom or re-operation). A secondary endpoint of Overall Survival (OS) was used and calculated from the date of diagnosis until the date of death. OS was used as a secondary analysis point because patients will have received additional salvage therapies at recurrence after RT/TMZ, The census date of 01.09.2010 was used for patients who were alive ($n = 4$) at the end of the study were excluded from OS analysis. The median overall survival of our cohort was 13.8 months and used to classify patients into two survival groups. Samples were then assigned to the sample groups of 'responders' ($n = 15$) or 'non-responders' ($n = 12$) for response cohort and long term survivors (LTS) ($n = 19$) and short term survivors (STS) ($n = 19$) for survival cohort (see Table 6.4).

In addition to this working patient cohort, a validation cohort (obtained from Australian Genomics and Clinical Outcomes of Glioma [AGOG]) including tumour specimens from 66 patients diagnosed with glioblastoma was also included in this study to confirm the biomarker discovery results. Patient's age, gender, diagnosis, vital status, type of surgery, number of surgeries, administered concurrent/adjuvant and salvage therapies were available for each patient.

Table 6.4 Classification of samples (training cohort)

PFS6 (n = 27)		OS (n = 38) (median survival = 13.83 months)	
Responders (n)	Non-responders (n)	LTS (n)	STS (n)
15	12	19	19

Abbreviations: LTS long-term survivors, NR: non-responders, OS: overall survival, PFS6: 6-months progression free survival, R: responders, STS: short-term survivors

6.2.6 Pre-processing of Data

Data pre-processing was conducted using Data manager Software 4 (Enterprise Edition Bio-Rad Laboratories, Fremont, CA, USA) and included calibration, baseline subtraction and normalisation processes. External mass accuracy was calibrated using ProteinChip All-In-One Protein Standard II (Bio-Rad, Hercules, CA, USA) (hirudin, 6.963 kDa; cytochrome C, 12.230 kDa; myoglobin, 16.951 kDa; carbonic anhydrase, 29.023 kDa; enolase, 45.67 kDa; albumin, 66.43 kDa and IgG proteins, 147.30 kDa) according to the manufacturer's protocol. Baseline subtraction was performed to increase comparability between spectra and to reduce variance within replicates. Peaks were detected in order to analyse differences in expression between protein profiles of

the samples. Common peaks across all spectra were clustered with the following settings: signal/noise (S/N) > 5; minimum peak threshold, 5% of all and mass error, 0.3%. After identification of significant peaks between groups, peaks were manually evaluated for quality and removed from the analysis if 50% of the samples were identified with S/N ratio < 5.0. Baseline subtraction and noise reduction were conducted before the normalisation of peak intensities. The majority of resolved peptides were found in the mass range below 30,000 Da. Ion noises of EAM interferes with the spectral region from 0 to 2,000 Da therefore this region was excluded from the analysis. In addition, mass region above 30,000 was not included in the analysis due to the weak signals with too small intensities. The protein intensities between 2,000 and 30,000 Da were normalised to the average total ion current (TIC) and normalisation factors (NF) were generated for each spectra. Spectra with a very high or low NF were eliminated from the analysis when the NF of a spectrum was deviated 2 standard deviations from the mean NF. Deviation of both spectra did not occur in any of the samples. Normalisation was applied separately for the data obtained from each ProteinChip surface. For the examination of variability between chips, coefficient of variation (CV%) was calculated by dividing the standard deviation (SD) by the mean peak intensity multiplied by 100%. Reproducibility of the ProteinChip assays was tested using ProteoMassTM Insulin MALDI-MS Individual Standard (Sigma-Aldrich, St Louis, MO, USA).

6.2.7 Statistical Analysis

After pre-processing of data, intensities of each peak in the group were averaged for each sample. Differences between samples and the two groups were determined by Expression Difference Mapping (EDM) using Data manager Software 4 (Enterprise Edition Bio-Rad Laboratories, Fremont, CA, USA). The software identified peaks using

Mann-Whitney U test in order to detect differentially expressed proteins in a data set which was not normally distributed. Results were interpreted as significant at p values less than 0.05 (Data manager Software 4, Enterprise Edition Bio-Rad Laboratories, Fremont, CA, USA). For each significantly differentially expressed protein, receiver operating characteristic (ROC) curves were constructed to obtain area under curve (AUC) values (SPSS Software Version 18.0, Chicago, IL). AUC values which ranges from 0.5 (no discrimination) to 1.0 (absolute prediction) were compared using the Hanley & McNeil method to evaluate discriminatory power of proteins and the significance of ROC curves (Hanley and Mcneil, 1982; Hanley and Mcneil, 1983). Correlation between potential markers was analysed by the calculation of Spearman's non-parametric correlation coefficients and $p < 0.05$ was considered significant (SPSS Software Version 18.0, Chicago, IL).

In the discovery of potential markers, significantly differentially expressed proteins with AUC value > 0.70 and p-value < 0.01 in response cohort and proteins with AUC value > 0.70 and p-value < 0.03 in survival cohort were considered for further investigation. As previously stated in Section 6.2.6, only the peaks which were qualified by manual evaluation were entered for binary logistic regression (BLR) analysis. Backward stepwise BLR procedure was applied to compute contribution of individual markers to the separation of the two test groups and also to estimate the complimentary performance of multiple biomarkers. Proteins with low discriminating ability were removed from the initially entered list of markers by backward stepwise elimination method. Hosmer and Lemeshow Goodness of Fit test was also applied to test whether the data analysed by BLR were a good fit. Validity of the BLR model was examined using goodness of fit test by calculation of observed and expected frequencies. Generated p-values greater than 0.05 indicate that the BLR model is a good fit and the

estimates of the suggested model fit the data adequately. All analyses were performed using the SPSS statistical package, Version 18.0 (SPSS Inc, Chicago, IL).

6.2.8 Identification of Candidate Biomarkers

After analysis and selection of biomarkers from the response and survival cohort, proteins were purified and identified by specific purification methods.

6.2.8.1 Ion-exchange Separation of proteins using Mustang S Filter Plate (Semi-purification)

Glioma tissue extracts were semi-purified using Mustang S cation-exchange AcroPrep 96 Filter Plate (350 μ l) (Pall Corporation, Port Washington, NY, USA). Based on the SELDI-TOF MS identified peak intensities of peak of interests, glioma samples expressing relatively higher levels of these proteins were selected for the purification. Sample fractionation was conducted by stepwise elution using seven chromatographic buffer systems with pH range between 4.0 and 9.0 (see Table 6.5). Filter membranes were pre-equilibrated with the addition of 100 μ l 50 mM sodium acetate, pH4.0 buffer into each well of 96 well plate which was then assembled into a Bioprocessor (Ciphergen Biosystems Inc.) to use automated shaking system for 10 minutes. Buffer was filtrated into a collection plate by applying a vacuum 25cm Hg on a multi-well plate vacuum manifold, (Pall Corporation, Port Washington, NY, USA) and discarded. This process was repeated two more times without the shaking step. Protein samples (60 μ l) were then diluted (1:10) in 50 mM sodium acetate, pH4 and 200 μ l of the prepared dilution was dispensed per well. The filter plate was incubated on the automated shaking system for one hour and flow-through containing unbound proteins was collected using vacuum manifold. Filtrates in the replicate wells were combined in a microcentrifuge tube. Incubation of the membranes in each well was continued by the

addition of other six buffers in the order of ascending pH range as detailed in Table 6.5 for 10 minutes for every step. Eluted samples (10µl/spot) were analysed on NP20 (normal phase) arrays by SELDI-TOF MS. After the spots were treated with the samples, arrays were allowed to dry and washed two times with MilliQ water thoroughly. EAM solution was applied in a similar manner as described in Section 6.2.3.1. Filtrates containing protein of interests were stored at -20⁰C for further purification steps.

Table 6.5 Buffers used for stepwise elution of crude protein samples

Elution Steps	pH Range	Buffers
1	pH 4.0	50 mM sodium acetate
2	pH 5.0	50 mM sodium acetate
3	pH 6.0	50 mM sodium acetate
4	pH 7.0	50 mM HEPES
5	pH 8.0	50 mM Tris-HCl
6	pH 9.0	50 mM Tris-HCl
7		50% ACN in MilliQ water

Abbreviations: ACN: acetonitrile, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Tris-HCl: tris-hydrochloride

6.2.8.2 Further Purification of Samples and Identification of Proteins

The semi-purified samples were further fractionated by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) (Model 510, Waters Corporation, Milford MA, USA) to obtain better purified subfractions containing proteins of interest. Semi-purified samples (2 ml) were injected onto the column and ran for 35 minutes. Gradient elution with a 15% ACN to 60% acetonitrile in 0.1% trifluoroacetic acid was performed on a 4.6 x 250mm Jupiter 5µm, 300Å C18 column (Phenomenex, Torrance, CA, USA) at 1.5ml/min flow rate. Then, the fractions (1.5 ml) were collected into

siliconised glass tubes (KIMBLE® Glass, Inc., Vineland, NJ, USA) using a fraction collector FRAC-100 (GE Healthcare, Buckinghamshire, UK). Prior to the HPLC analysis, the tubes were coated with dimethyldichlorosilane Coatasil Glass Treatment Solution (Lomb Scientific-Thermo Fisher, NSW, Australia) and allowed to dry in a fumehood for 30 minutes. Collected fractions were then analysed on NP20 arrays and the fraction containing the purified protein was lyophilised using Cryodos Freeze Drier (Telstar Industrial, Terrassa, Spain). Freeze-dried proteins were reconstituted in PBS and samples were prepared as detailed in Table 6.7 and then heated at 70 °C for 10 minutes prior to loading gel. SeeBlue Pre-Stained Plus (Invitrogen, Carlsbad, CA, USA) and unstained Protein Standard, BenchMark™ Protein Ladder (Invitrogen, Carlsbad, CA, USA) were loaded across the gel to estimate molecular weights of proteins. Two different pre-cast, 12% NuPAGE Novex (Invitrogen, Carlsbad, CA, USA) and 4-20% Mini-PROTEAN TGX (Bio-Rad Laboratories, Hercules, CA, USA) gels were used to obtain sharp bands by electrophoresis (see Table 6.6). To detect purified protein bands, a fluorescent SYPRO ruby protein stain (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's instructions. The gels were handled meticulously to minimise the keratin contamination. Following incubation with SYPRO Ruby, stained gels were visualised by UV fluorescence on a UV transilluminator (UVP Inc., San Gabriel, CA, USA). Protein bands corresponding proteins of interest were excised from the gel with a sterile scalpel and placed carefully in microcentrifuge tubes. For protein identification, the gel sections were sent to the Bio-analytical Mass Spectrometry Facility (BMSF), University of New South Wales, Sydney, Australia.

Table 6.6 Electrophoretic conditions used for the separation of proteins

Gel Types	Buffers	Conditions
NuPAGE Novex 12 % Bis-Tris Gel	1X The NuPAGE® MES SDS Running Buffer	180 V for 40 minutes
4-20% Mini-PROTEAN TGX Gel	1X Tris-Glycine Running Buffer (see Appendix B)	180 V for 45 minutes

Abbreviations: MES: 2-(N-morpholino)ethanesulfonic acid, SDS: sodium dodecyl sulfate, V: volts

6.2.9 Validation of Identified Biomarkers by Western Blotting

Protein samples were extracted from five commercial glioma cell lines (T98G, A172, M059K, U-87 MG, M059J), one commercial recurrent glioma cell line (DBTRG) and five glioma neural stem cell (GNS) cultures derived from primary glioblastomas (HWK1, PB1, WK1, RN1, BAH1). Protein samples were denatured in sample buffer containing NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) and NuPAGE Sample Reducing Buffer (10X) (Invitrogen, Carlsbad, CA, USA) as detailed in Table 6.7. Prepared samples (30µg) were electrophoresed and separated proteins on the gel were transferred to an Amersham™ Hybond™ ECL Nitrocellulose Membrane (GE Healthcare, Buckinghamshire, UK) as previously described in Section 2.5.3.2 and Table 2.11, Materials and Methods Chapter 2. Membranes were blotted using Alpha Defensin (1+2+3) Polyclonal Goat Antibody (Abcam, Cambridge, UK) for DEFA3, MIF Polyclonal Goat Antibody (R&D Systems, Minneapolis, MN, USA) for MIF and S100A8 Polyclonal Sheep Antibody (R&D Systems, Minneapolis, MN, USA) for S100A8 under optimised conditions detailed in Table 2.12, Materials and Methods, Chapter 2. The protocol described in Section 2.5.3.2 was followed to develop and visualise blots.

Table 6.7 Components of protein mix for gel loading

Reagents	Volume (µl)
Protein in lysis buffer	x
PBS solution	13-x
NuPAGE LDS Sample Buffer	5
Nu PAGE Sample Reducing Buffer (10X)	2
Final volume	20

6.2.10 Protocol for Immunohistochemical Staining of DEFA3, S100A8 and MIF protein expression

Immunohistochemistry was performed by Dr Fei Shang, at Histology and Microscopy Unit, Faculty of Medicine, UNSW. Paraffin-embedded sections (4 µm) of glioblastoma tissue (n=71) and positive control tissues; human tonsil (FFPE) for DEFA3, human spleen (FFPE) for S100A8 and human breast cancer tissue (FFPE) for MIF, were deparaffinised and dehydrated. The sections were treated with peroxide block reagent (Bond polymer Refine Detection Kit, Leica Microsystems, VIC, Australia) to block endogenous peroxidatic activity. The sections were then immunostained with DEFA3 (T-1034; BMA Biomedicals, Augst, Switzerland), S100A8 (ab92331; Abcam, Cambridge, UK) or MIF (MAB289; R&D Systems, Minneapolis, MN, USA) antibodies (listed in Table 6.8) on Bond X automated immunostainer (Leica, Microsystems, VIC, Australia) for 30 minutes. Prior to peroxidase blocking the sections stained for DEFA3 were treated with proteinase K for 7 minutes in place of microwave heat treatment step. Negative controls were incubated with the labelled secondary antibodies only. Immunostaining was developed with DAB-Chromogen for 10 minutes and

counterstained with haematoxylin to allow nuclei visualisation (Bond polymer Refine Detection Kit, Leica Microsystems, VIC, Australia). Sections cut in parallel to the antibody-treated sections were stained by H&E for the identification of tissue areas clearly. IHC staining is currently under evaluation by a qualified neuropathologist (Dr Michael Rodriguez).

Table 6.8 Conditions of staining for the antibodies used in immunohistochemistry

Antibodies	Dilutions
DEFA3 (monoclonal mouse)	1:10000
S100A8 (monoclonal rabbit)	1:2000
MIF (monoclonal mouse)	1:8000

6.3 RESULTS

6.3.1 Optimisation of Lysis Buffer Used in Protein Extraction

Final concentration of DTT in protein samples was 64.8 mM after extraction. DC™ Protein Assay is only compatible with maximum 1 mM of DTT. Therefore, dilutions (1:100) of samples were prepared using the lysis buffer with and without DTT. BSA standards were also prepared in DTT containing and DTT-free lysis buffers. The final concentration of DTT in the diluted BSA standards was 0.64mM which was tolerable for protein assay. DTT-free buffer gave rise to a R^2 value of 0.972 which showed a better fitting trendline than DTT containing buffer (see Figure 6.2). A non-linear trendline ($R^2 = 0.959$) was constructed when DTT containing buffer was used to generate the standard (see Figure 6.3). Protein concentrations of samples were predominantly detected below the least concentrated point on the standard curve when 100 times less concentrated forms of the samples were used as a result of limiting the DTT. Therefore optimal condition for the preparation of samples in lysis buffer was excluding DTT from the buffer until the analysis.

Following optimisation of protein lysis buffer, proteins were extracted in DTT-free lysis buffer and 1 in 30 dilutions of samples were analysed by DC™ Protein Assay. An additional concentration standard (0.0625 mg/ml) was included in the standard curve (below 0.125 mg/ml) in order to define curve more precisely and increase the accuracy of estimates. Standard curves used for the quantification of proteins from cell lines and tumour tissue samples are demonstrated in Figure 6.4 and Figure 6.5, respectively.

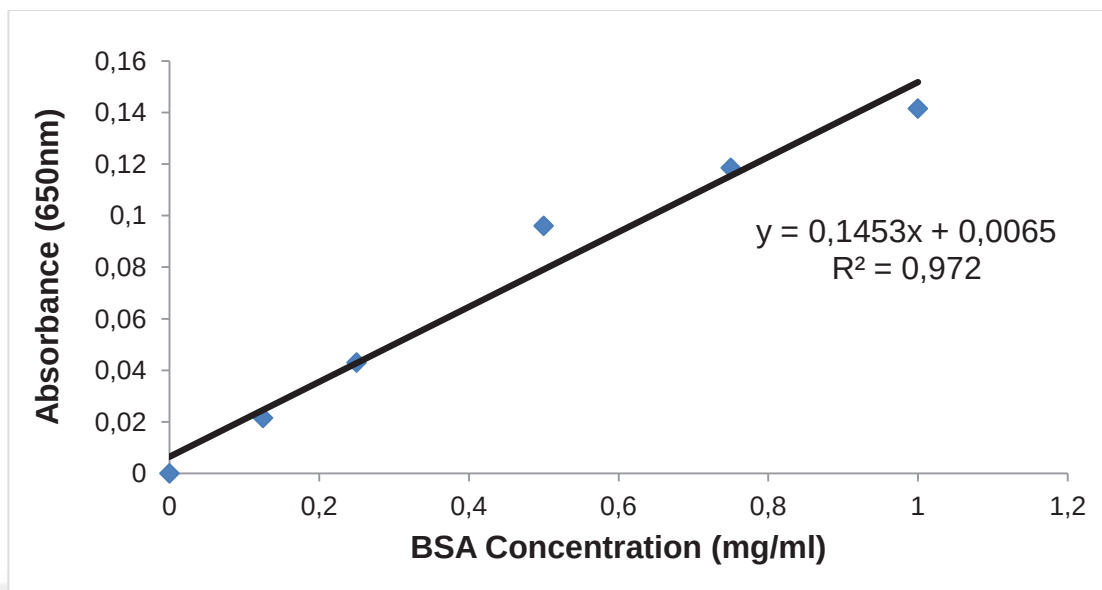


Figure 6.2 Standard curve of BSA in lysis buffer without DTT

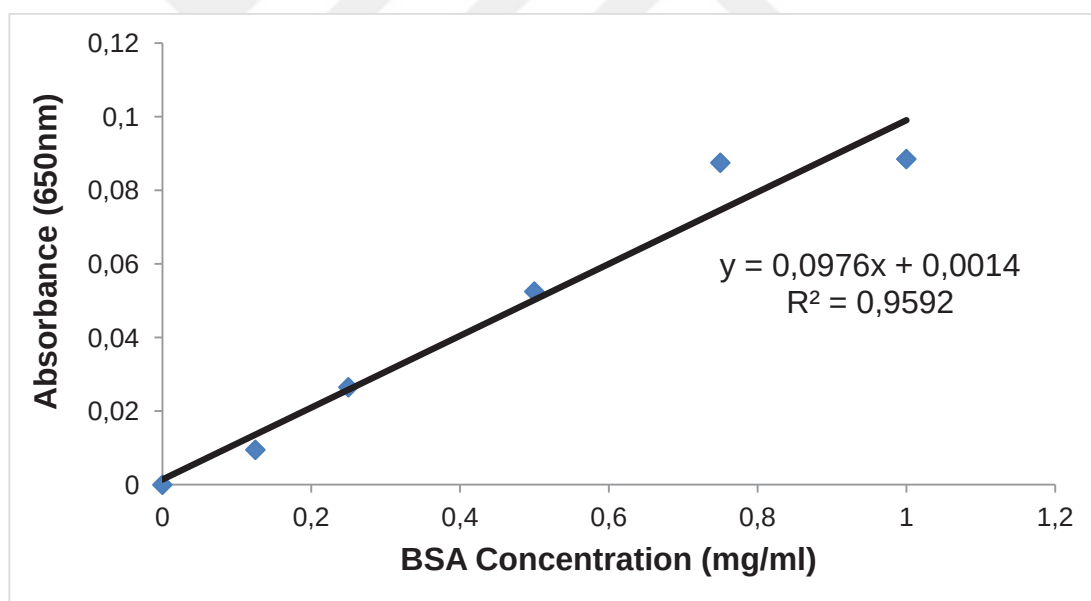


Figure 6.3 Standard curve of BSA in lysis buffer with DTT

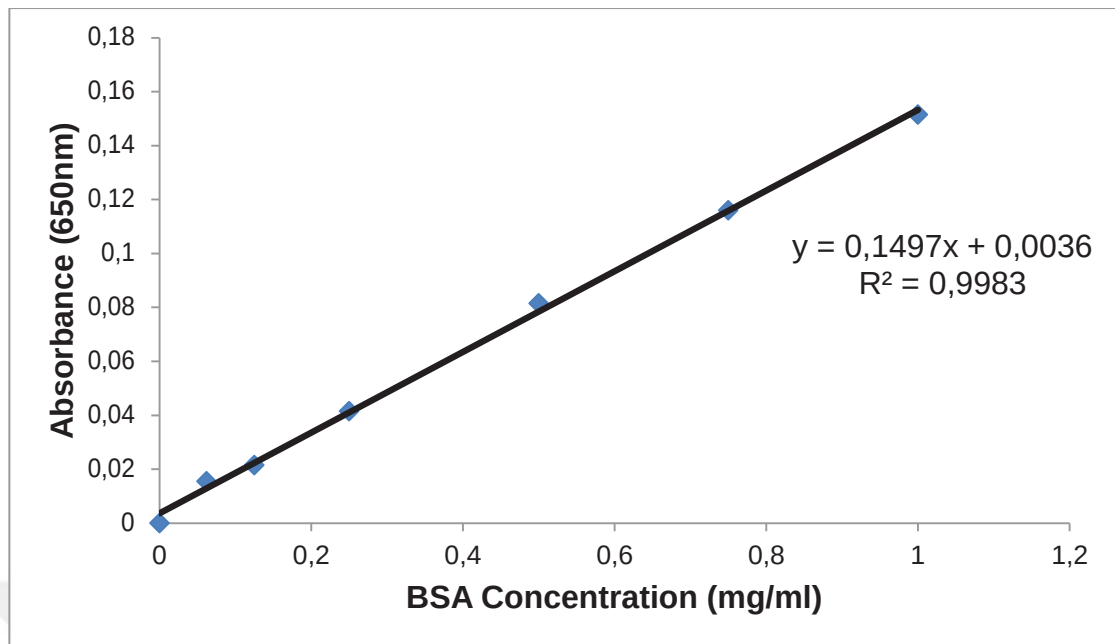


Figure 6.4 Standard curve of BSA in lysis buffer without DTT used for the quantification of proteins from the cell lines

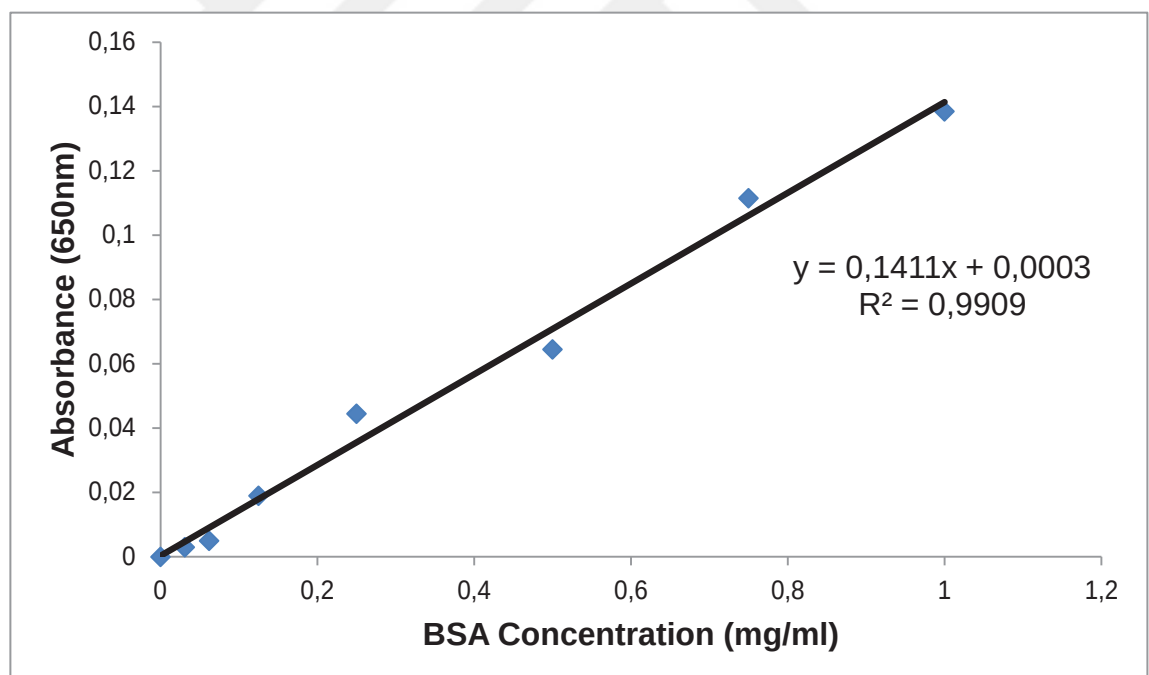


Figure 6.5 Standard curve of BSA in lysis buffer without DTT used for the quantification of proteins from the tumour tissue samples

6.3.2 Optimisation of Laser Intensities and Binding Buffers

Laser intensities of 1200 nJ, 2000 nJ, 1600 nJ and 1900 nJ were validated for ProteinChip arrays; H50, Q10, CM10 and IMAC, respectively. Warming shots used prior to data collection is detailed in Table 6.9. Tris-HCl buffers (50mM) with different pHs (pH 7.5, pH 8.0 and pH 8.5) were used to optimise a binding buffer for Q10 array (see Figure 6.6). Improved reproducibility of peaks was observed in both low and high mass range when 50 mM Tris-HCl pH 8.5 was applied on Q10 array spots. The buffer at pH 8.5 was selected for the further applications on Q10 array. To select the most efficient buffer and pH conditions for CM10 arrays, 50 mM sodium acetate and 50mM ammonium acetate buffers with varying pH range (pH 4.0, pH 5.0 and pH 6.0) were analysed (see Figure 6.7). More reproducible peaks in a wide mass range were obtained with sodium acetate pH 4.0 in comparison to the peak reproducibility of other binding buffers. Sodium acetate at pH 4.0 was confirmed to be used for the full profiling of samples with CM10 arrays. Optimised buffers are listed in Table 6.10 for the applications using H50 and IMAC arrays.

Table 6.9 Conditions optimised for laser intensities

ProteinChip Arrays	Warming Shots (nJ)	Data Shots (nJ)
H50	1320	1200
Q10	2200	2000
CM10	1760	1600
IMAC	2090	1900

Abbreviations: CM10: cation exchange, H50: hydrophobic, IMAC: immobilised metal affinity capture, nJ: nanojoule, Q10: anion exchange

Table 6.10 Optimal pH Conditions for array binding buffers

ProteinChip Arrays	Binding Buffers
H50	10% Acetonitrile, 0.1% TFA (Recommended)
Q10	50 mM Tris-HCl, pH 8.5 (Optimised)
CM10	50 mM Sodium Acetate, pH 4.0 (Optimised)
IMAC	1X PBS, pH 7.2 (Recommended)

Abbreviations: CM10: cation exchange, H50: hydrophobic, HCl: hydrochloric acid, IMAC: immobilised metal affinity capture, PBS: phosphate buffered saline, Q10: anion exchange, TFA: trifluoroacetic acid

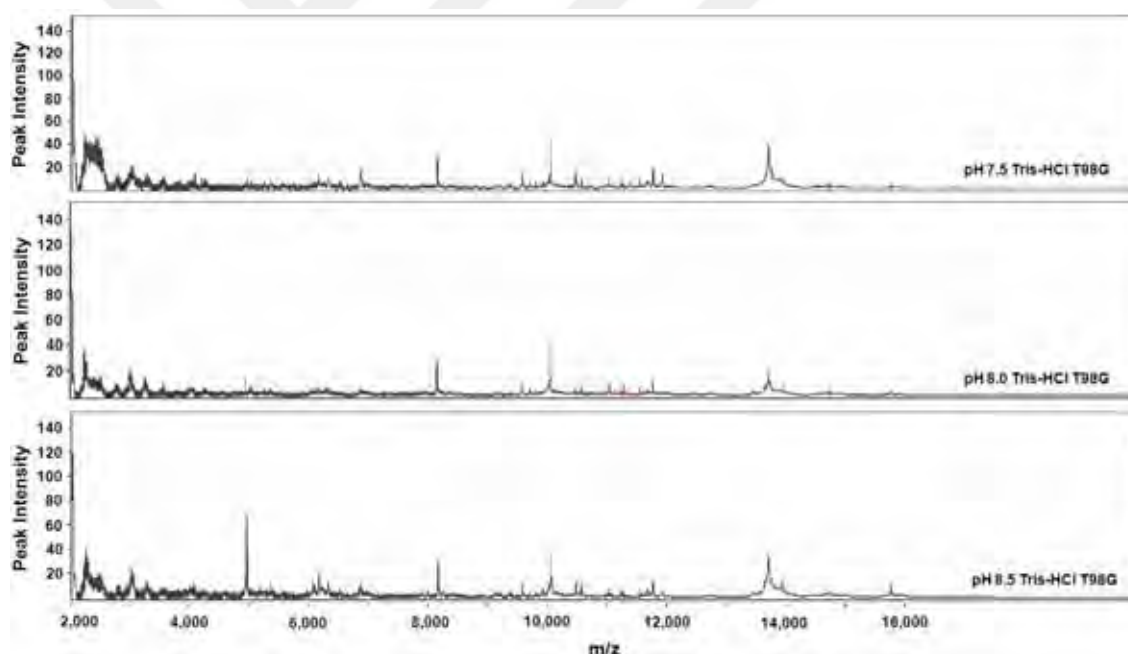


Figure 6.6 Optimisation of buffers using T98G cell line protein extracts for Q10 array

A graphical representation of the spectra from the optimisation of buffers with different pHs.

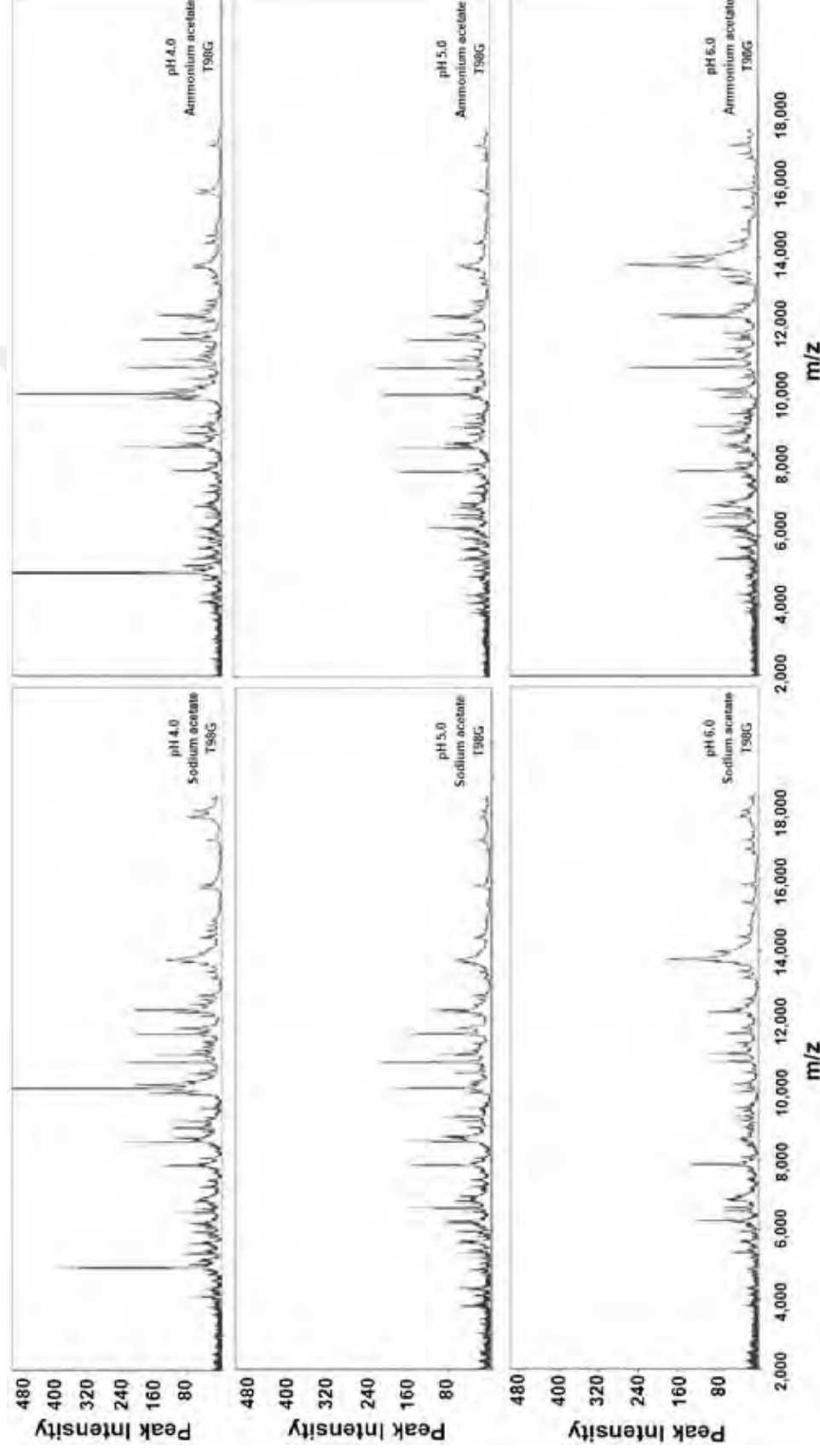


Figure 6.7 Optimisation of buffers using T98G cell line protein extract for CM10 array

The spectra from the optimisation of two different buffers (50 mM sodium acetate and 50mM ammonium acetate) with pH 4.0, 5.0 and 6.0.

6.3.3 SELDI-TOF MS Data Analysis

Pre-processing of data was maintained as described in Section 6.2.6. Variation between chips of each ProteinChip array type was evaluated by analysing 5 pmol/μl dilutions of insulin standard. Inter-chip variability was established as a result of the calculation of Percent Coefficient of Variation (CV%) from peak intensities of ProteoMass™ Insulin Standard. CVs of 4.9, 16.42, 11.97 and 16.35% were determined for H50, Q10, CM10 and IMAC, respectively. Q10 and IMAC arrays showed particularly higher CVs than H50 array. H50 spectra were highly reproducible, with CV under 5%.

Normalisation was conducted based on the computed NFs for each spectrum and calculation of standard deviations of NFs between samples. Seven out of 216 spectra for response and 10 out of 304 spectra for survival cohort were eliminated from the analysis (see Table 6.11). In none of those cases both duplicates were eliminated. Subsequently, in the duplicate spectra of a subject, the intensities of the detected peaks with the same mass were averaged. For samples with one spectrum left, only peak intensities of that spectrum were used for analysis.

Table 6.11 Summary of the normalisation analysis

ProteinChip Arrays	Response Cohort			Survival Cohort		
	Nr of Samples	Nr of Spectra	Excluded Spectra	Nr of Samples	Nr of Spectra	Excluded Spectra
H50	27	54	2	38	76	3
Q10	27	54	1	38	76	2
CM10	27	54	2	38	76	2
IMAC	27	54	2	38	76	3
Total		216	7		304	10

Abbreviations: CM10: cation exchange, H50: Hydrophobic, IMAC: immobilised metal affinity capture, Q10: anion exchange

Following the normalisation process, clusters of common peaks were generated using Data manager Software 4. Twenty-seven patients from our total patient cohort were grouped as either responders (PFS ≥ 6 months, n = 15) or non-responders (PFS < 6 months, n = 12). In 54 protein profiles collected in duplicate from 27 samples, 225, 88, 198 and 123 common peaks were identified for H50, IMAC, Q10 and CM10 chips, respectively (see Table 6.12). In combination of data from all chip surfaces, 44 significantly differentially expressed proteins were detected between the two PFS groups using a Mann-Whitney U test at $p < 0.05$ level. While 26 of these proteins were down-regulated in non-responders, 18 proteins were found to be up-regulated (see Appendix D).

Thirty-eight out of 42 patients could be categorised into Long Term Survivors (LTS) (n = 19) and Short Term Survivors (STS) (n = 19) subgroups. The peak clusters 68, 81, 76 and 43 were identified common to the both patient groups for namely H50, Q10, CM10 and IMAC arrays. Thirty-nine protein peaks were found differentially expressed between groups (see Table 6.12). Seven proteins of the 39 identified peaks were down-regulated while 32 protein peaks were over-expressed in LTS group (see Appendix D).

The combination of peak clusters captured by H50, CM10 and IMAC arrays formed 86% of all common peaks detected in the PFS6 response cohort. Similarly in survival cohort (OS), 84% of all common peak clusters were detected by H50, Q10 and IMAC arrays. While Q10 surface exhibited a poor efficiency in identifying common peaks in response cohort, analysis in survival cohort displayed high detection performance of Q10 array with 30% of all common peaks.

Table 6.12 Results for peak clustering of sample groups (Response and Survival)

ProteinChip Arrays	Response Cohort		Survival Cohort	
	Common Peaks	Nr of Significant peaks (< 0.05)	Common Peaks	Nr of Significant peaks (< 0.05)
H50	225	13	68	1
Q10	88	16	81	3
CM10	123	6	76	30
IMAC	198	9	43	5
Total Nr of Peaks	634	44	268	39

Abbreviations: CM10: cation exchange, H50: Hydrophobic, IMAC: immobilised metal affinity capture, $p < 0.05$ non-parametric Mann-Whitney U test, Q10: anion exchange

6.3.4 Selection of Significant Proteins and BLR Analysis

Peak clusters with higher p-value, AUC and peak quality were subjected to backward stepwise BLR analysis in order to generate a reliable biomarker panel and evaluate the combined discrimination power of selected biomarker candidates. Peak overlaps between four different ProteinChip arrays were observed (m/z, 10850 on H50 and m/z 10836 on Q10) (see Appendix D) and the peak with higher significance was included in the analysis. Numbers of significantly up-regulated proteins in non-responder, responder, STS and LTS groups were 4, 9, 4 and 3, respectively (see Table 6.13). Selected proteins consisted of a combination of proteins detected on all of the four array surfaces but most prominently on the Q10 and CM10 surfaces. Discriminatory power of the selected peaks identified in the analysis of response groups ($AUC > 0.75$, $p \leq 0.01$) was higher than the peaks selected from the significantly differentially expressed peaks of survival groups ($AUC > 0.70$, $p \leq 0.03$). m/z ratios of the highly significant

proteins included in BLR analysis were within the mass range below 20 kDa.

Table 6.13 Significantly differentially expressed proteins analysed by BLR

		m/z	Array Type	Mean Intensity		P-value	AUC
				NR	R		
Analyses of Samples in Response Cohort	Up-regulated Proteins in Non-responders	10850.804	H50	29.306	11.736	0.001	0.878
		4439.449	H50	12.183	5.437	0.010	0.794
		10247.184	CM10	39.693	17.531	0.001	0.889
		12361.517	CM10	22.406	11.632	0.005	0.822
	Up-regulated Proteins in Responders	7934.333	Q10	106.473	176.602	0.000	0.917
		15865.953	Q10	254.388	459.882	0.002	0.856
		16012.455	Q10	71.944	128.173	0.005	0.822
		16076.491	Q10	77.211	142.898	0.005	0.822
		16872.066	Q10	37.153	78.421	0.005	0.817
		16129.833	Q10	66.813	125.376	0.005	0.817
		17170.242	Q10	19.381	42.689	0.004	0.828
		17874.649	Q10	6.634	17.531	0.007	0.806
		4255.869	Q10	54.011	78.500	0.007	0.809
		m/z	Array Type	Mean Intensity		P-value	AUC
				STS	LTS		
Analyses of Samples in Survival Cohort	Up-regulated Proteins in STS patients	3678.923	Q10	61.772	27.992	0.005	0.765
		12398.433	CM10	61.772	27.992	0.005	0.767
		11560.080	CM10	16.022	9.175	0.014	0.734
		11608.014	CM10	24.286	9.906	0.024	0.715
	Up-regulated Proteins in LTS Patients	7934.333	Q10	127.725	162.431	0.015	0.731
		15866.337	Q10	12.435	23.816	0.015	0.731
		5191.461	IMAC	117.663	225.405	0.020	0.720

Abbreviations: AUC: area under the Receiver Operating Characteristic (ROC) curve, CM10: cation exchange, H50: Hydrophobic, m/z: mass to charge, IMAC: immobilised metal affinity capture, LTS: long term survivors, NR: non-responders, p-value: non-parametric Mann-Whitney U test, Q10: anion exchange, R: responders, STS: short term survivors

6.3.5 Development of Candidate Biomarker Panels by BLR Analysis in Response and Survival Cohorts

BLR analysis of selected peaks revealed biomarker panels consisting three, five, two and one up-regulated protein(s) (see Figure 6.8) in non-responder, responder, STS and

LTS groups, respectively. After BLR analysis, these reduced panels of significantly up-regulated proteins in non-responders (10247, 12361 and 10850 Da) and responders (m/z 7934, 15865, 16076, 16129 and 4255 Da) were found to be the best models which were capable of distinguishing one group from another with a 100% sensitivity and specificity (see Figure 6.8, E and F). Both panels with 3 and 5 biomarkers in response groups resulted in a 100% correct classification of 12 patients in the non-responder and 15 patients in the responder patient group. However, combination of two up-regulated proteins (12398, 3678 Da) in STS cohort could correctly classify 84.2% of patients into either STS or LTS group (see Figure 6.8, G). In the BLR model generated for the up-regulated proteins in LTS group, discriminatory power of the single 15866 Da biomarker was detected with 68.4% sensitivity, 84.2% specificity and overall with a low classification percentage 76.3% (see Figure 6.8, H).

The Hosmer & Lemeshow Goodness of Fit test was also applied in order to establish the best fitting BLR model by determining significance (Chi Square, $p < 0.05$; Hosmer & Lemeshow sig., $p > 0.5$) of the combined peaks at different steps of the analysis. Each step of regression analyses of the significantly up-regulated proteins in response groups presented perfect fit (Chi Square, $p < 0.000$; Hosmer & Lemeshow sig., $p = 1.000$) of the logistic model with the data (Figure 6.8, A and B). Good fitting of the model for up-regulated proteins in STS was observed at the first (Chi Square, $p = 2.256$); Hosmer & Lemeshow sig., $p = 0.972$) and second (Chi Square, $p = 1.962$); Hosmer & Lemeshow sig., $p = 0.982$) steps of the BLR analysis. Whereas, the following BLR step with low significance (Chi Square, $p = 5.621$ and Hosmer & Lemeshow sig., $p = 0.690$) determined the general fit of the model to the data as inadequate (Figure 9, C). At all

stages of the BLR analysis of up-regulated proteins in LTS group represented an inapplicable quality of model adjustment with Chi Square p-values greater than 10.000 and Hosmer & Lemeshow sig., $p < 0.5$ (Figure 6.8, D).

Among these models, combination of three up-regulated proteins in the responder patient cohort were identified as the most appropriate candidate biomarker set with the remarkable sensitivity, specificity and also with an excellent fitting of the BLR model with the dataset.



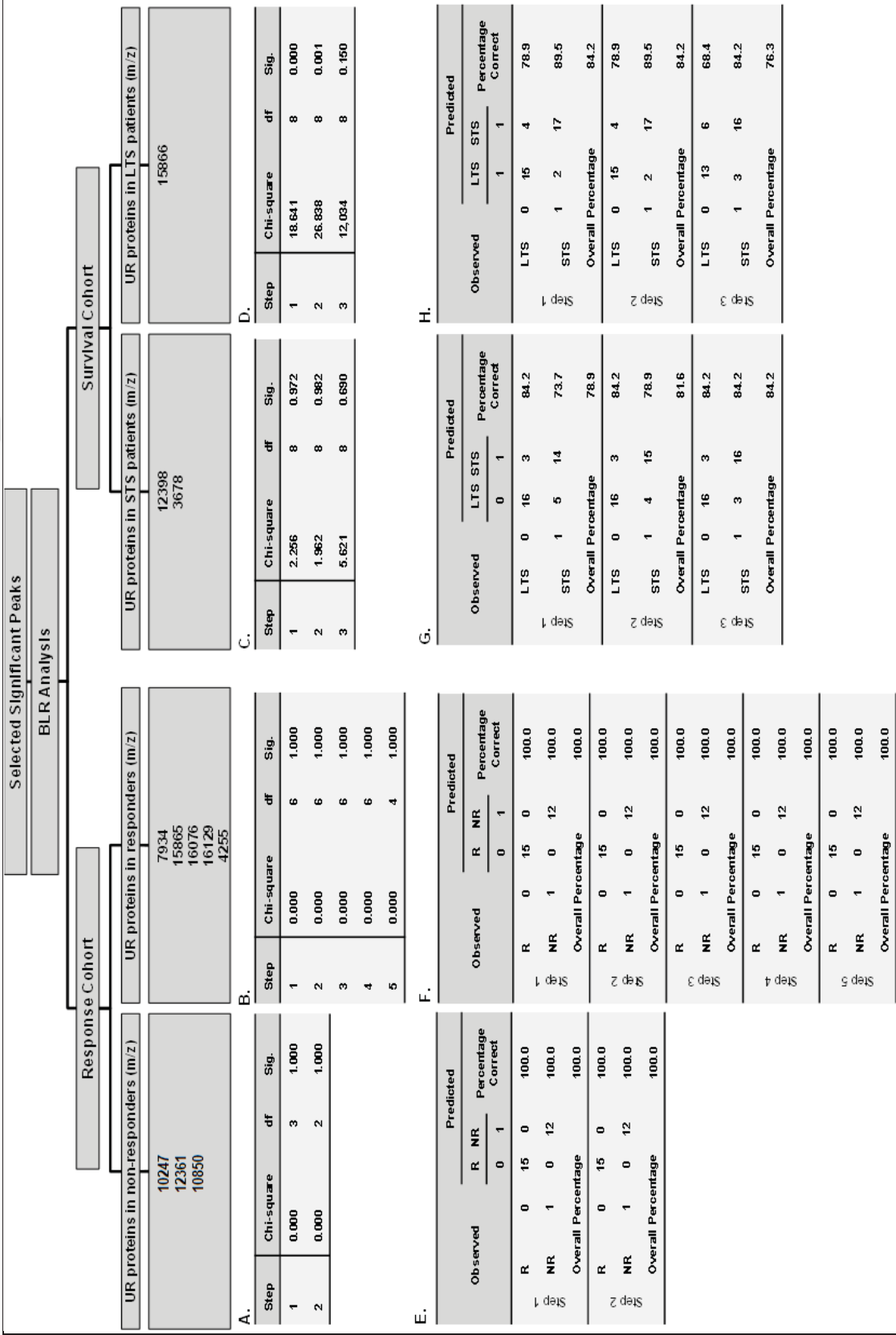


Figure 6.8 Candidate biomarker panels identified in patient subgroups by BLR Analysis

A, B, C and **D** represent Hosmer & Lemeshow Goodness of Fit Test results. **E, F, G** and **H** indicate classification tables used to compute sensitivity and specificity of the candidate biomarkers.

(E and F) Analysis of significant proteins using BLR yielded powerful biomarker sets of three and five with 100% sensitivity and specificity to discriminate non-responders from responders. **(A and B)** In addition to discriminatory power of identified proteins, the best model fit (Chi Square, $p < 0.000$; Hosmer & Lemeshow sig., $p = 1.000$) was observed at all steps of BLR analysis. **(G and H)** However, overall classification ability of the biomarkers revealed by BLR analysis in survival cohort was found relatively poor (84.2% in STS, 76.3% in LTS) compared to the panels identified for response. **(C)** Evaluation of the BLR model fit with the survival data sets indicated a low significance with an overall Chi Square, $p = 5.621$ and Hosmer & Lemeshow sig., $p = 0.690$ in STS. **(D)** Non-significant results of Chi Square (p-values 10.000) and Hosmer & Lemeshow test ($p < 0.5$) in LTS group resulted in an inapplicable BLR model.

Abbreviations: BLR: Binary Logistic Regression, df: degrees of freedom, LTS: Long term survivors, m/z: mass to charge, NR: non-responders, R: responders, Sig.: Hosmer & Lemeshow Goodness of Fit Test significance (cut-off point 0.5), STS: Short term survivors patients, UR: up-regulated

Figure 6.9 displays an overlay plot of representative SELDI-TOF MS spectra collected from the entire responder and non-responder patient cohort. 10-12.8 kDa of the spectra is plotted with molecular mass on the x-axis and the peak intensity on the y-axis. Spectra in red indicate the spectra from non-responders and the spectra of responders are shown in green. The peaks with significantly up-regulated expression levels in non-responders corresponding to 10.2 and 12.3 kDa proteins were detected on CM10 array surface (indicated by arrows in Figure 6.9). Both proteins were present in the panel of biomarkers up-regulated in non-responders shown in Figure 6.8 earlier.

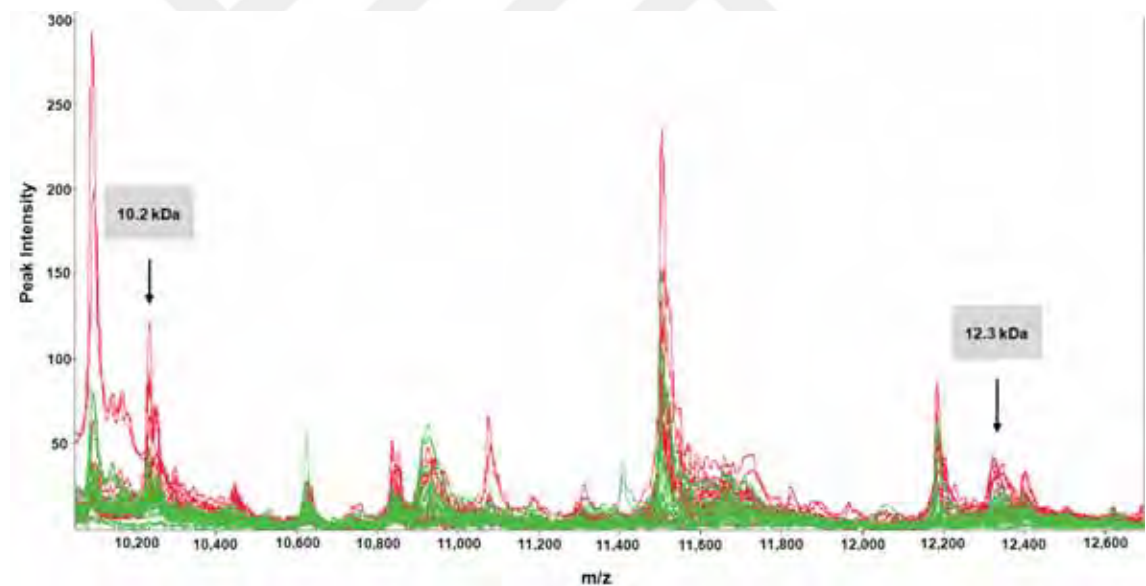


Figure 6.9 A representative overlay spectra of tumour tissue sample profiles of responders versus non-responders analysed by CM10 arrays

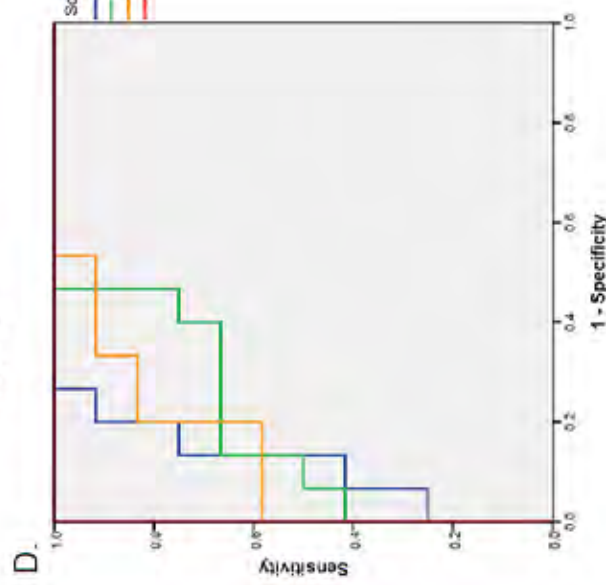
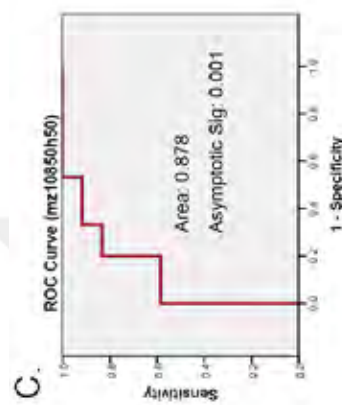
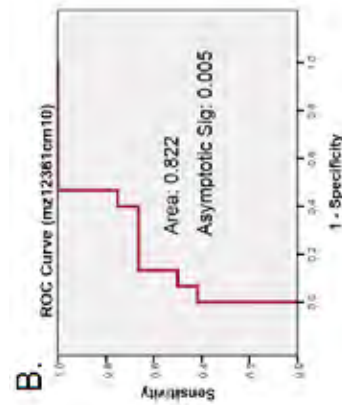
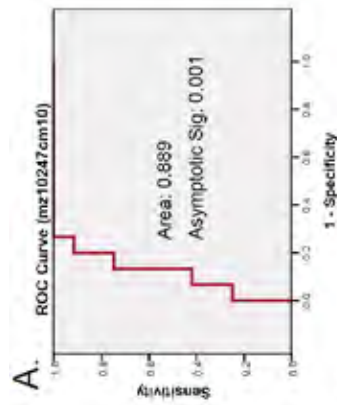
The intensity of peaks is plotted versus the molecular mass range between 10 and 12.8 kDa. The spectra highlighted in red are from non-responders ($n = 12$) and the green spectra are protein profiles of responders ($n = 15$).

6.3.6 ROC Curve Analysis and Spearman Correlation of Three Biomarkers Up-regulated in Non-responder Patient Group

6.3.6.1 ROC Curve Analysis of Selected Three Biomarkers in Response Cohort

In light of the results from BLR analysis described in Section 6.3.3, ROC curve analysis was performed for biomarkers individually and in combination. Predicted probability calculated by BLR analysis exhibited the combined power of selected biomarkers in discriminating one patient group from the other. Predictive powers of individual biomarkers, 10247 Da, 12361 Da, 10850 Da were indicated by AUC values 0.889, 0.882, 0.878, respectively (shown in Figure 6.10 A, B and C, summarised in E). All points were found within the range between 0.8 and 0.9 with the asymptotic significance of < 0.01 indicating a good discriminating ability of each biomarker. AUC value of 1.000 (Asymptotic sig = 0.00 and 95% confidence interval 1.000-1.000) (see Figure 6.10 E) computed by the combination of the three biomarkers which demonstrated the excellent predictive ability of the model. The selected biomarkers in combination achieved a ROC curve located at 1.0 on vertical axis and at 0.0 on horizontal axis indicating an outstanding discriminating ability (Figure 6.10 D).

Furthermore in Figure 6.10, the expressions of these proteins were demonstrated by a heat map (E) showing relative protein intensity of each patient sample in columns and selected biomarkers in rows. Up-regulation of proteins compared to the mean intensity value is indicated by red while green represents down-regulated expression compared to the mean. Black indicated no expression change from the mean value. Protein expression patterns of biomarkers (10.2, 12.3 and 10.8 kDa) visualised on heat map confirmed relatively high expression of the proteins in the non-responders compared to the responder patient group.



E.

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Lower Bound	Upper Bound
10247cm10	0.889	0.066	0.001	0.720	1.000
12361cm10	0.822	0.080	0.005	0.665	0.979
10850h50	0.878	0.066	0.001	0.742	1.000
Predicted probability	1.000	0.000	0.000	1.000	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

F.



Figure 6.10 ROC curve analysis, Hanley & McNeil test including 95% CIs and representative heat map showing expression levels for the three biomarkers identified in Response cohort

AUC values; 0.889, 0.882, 0.878 and 1.000 represent performance(s) of biomarkers (A) 10247 Da, (B) 12361 Da, (C) 10850 Da and (D-E) biomarkers in combination, respectively, to classify responder and non-responder patients. Combinatory analysis of biomarkers resulted in a perfect (D) ROC curve possessing high discriminatory power with (E) Asymptotic sig < 0.01 and 95% confidence interval, 1.000-1.000. (F) Heat map of protein expression patterns of each biomarkers with columns displaying patients (n = 27) in response cohort and row representing m/z of three biomarkers. Up-regulated expression is shown in red, down-regulated expression in green and black indicates no different expression from the mean value

Abbreviations: Area: AUC (area under the ROC curve), Asymptotic 95% CI: 95% confidence interval, Asymptotic Sig: Hanley & McNeil method, ROC: receiver operating characteristics

Note: The AUC results were considered excellent between 0.9-1, good between 0.8-0.9, fair between 0.7-0.8, poor between 0.6-0.7 and values between 0.5 and 0.6 represent low power of protein to discriminate two groups. Values ≤ 0.5 means no discriminatory power of protein.

6.3.6.2 Spearman Correlations between Selected Three Biomarkers in Response Cohort

Spearman correlation coefficients were calculated to examine the relationship between expression patterns of 10247, 12361, 10850 Da proteins. Correlation coefficient values close to 1 represent the significant association between the expressions of biomarkers. A strong positive correlation ($R = 0.667$, $p < 0.000$) existed between 10247 and 10850 Da proteins. Protein expression of 10247 and 12850 Da proteins were also significantly correlated ($R = 0.429$, $p < 0.025$) in response cohort. Although a positive (0.206) correlation coefficient was found between 12361 and 10850 Da proteins, the significance of the correlation was greater than 0.05, suggesting a lack of direct association between the expression patterns of these biomarkers (see Table 6.14).

Table 6.14 Spearman correlation for three biomarkers identified in response cohort

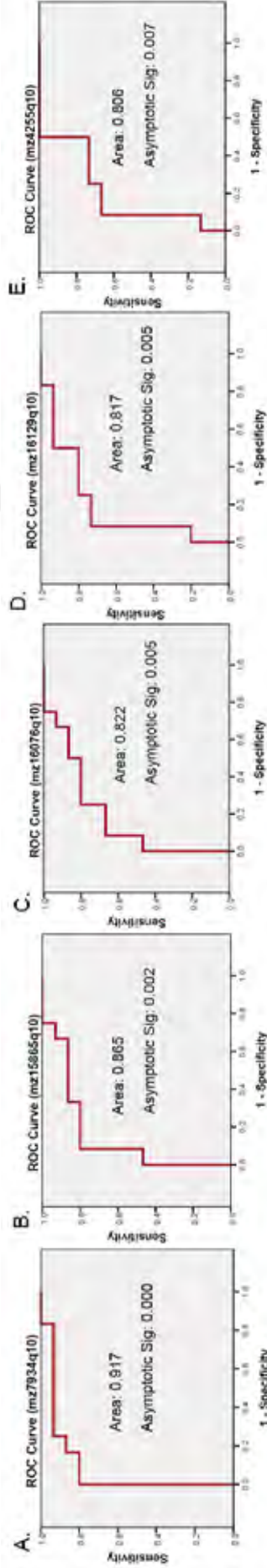
			10247cm10	12361cm10	10850h50
Spearman's rho	10247cm10	Correlation Coefficient	1.000	0.429*	0.667**
		Sig. (2-tailed)		0.025	0.000
		N	27	27	27
	12361cm10	Correlation Coefficient	0.429*	1.000	0.206
		Sig. (2-tailed)	0.025		0.303
		N	27	27	27
	10850h50	Correlation Coefficient	0.667**	0.206	1.000
		Sig. (2-tailed)	0.000	0.303	
		N	27	27	27

* Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed), Correlation coefficient (R): 0 = no association between variables, 1 = significant association between variables, Abbreviations: N: number of samples

6.3.7 ROC Curve Analysis and Spearman Correlation of Five Biomarkers Up-regulated in Responder Patient Group

6.3.7.1 ROC Curve Analysis of Selected Five Biomarkers in Response Cohort

ROC curves were computed to assess predictive ability of individual markers, displaying AUC values of 0.917, 0.865, 0.822, 0.817 and 0.806 for 7934, 15865, 15076, 16129 and 4255 Da proteins, respectively. Predictive ability of each biomarker indicated high power ($AUC > 0.8$) to discriminate two groups of patients in response cohort. Asymptotic significance and 95% confidence intervals were constructed to measure precision of prediction for each protein and shown in Figure 6.11, G. An acceptable narrower range of confidence interval between 0.6 and 1.0 was observed for the entire biomarker panel except for 7934 Da protein with a wide confidence interval (0.0-1.0) indicating poor precision. However, AUC value of 1.000 (Asymptotic sig. = 0.000, 95% confidence interval = 1.000-1.000) for the final prediction model calculated by the cumulative contribution of ROC curves of five biomarkers suggested the best discriminating power. Significant combinatorial power of the selected five biomarkers is illustrated as a ROC curve highlighted in red in Figure 6.11, F.



G.

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% CI	
				Lower Bound	Upper Bound
7934q10	0.917	0.59	0.000	0.000	1.000
15865q10	0.856	0.75	0.002	0.707	1.000
16076q10	0.822	0.80	0.005	0.665	0.980
16129q10	0.817	0.86	0.005	0.647	0.986
4255q10	0.806	0.89	0.007	0.632	0.979
Predicted probability	1.000	0.000	0.000	1.000	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

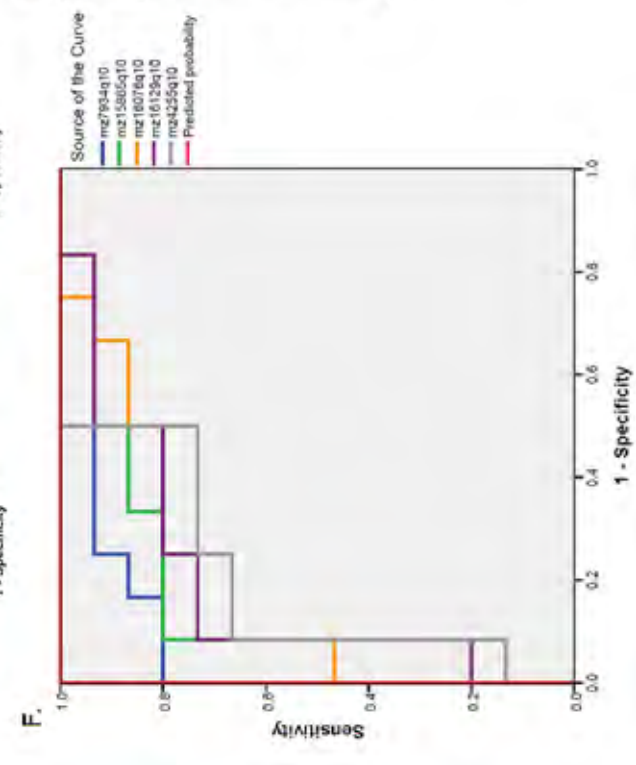


Figure 6.11 ROC curve analysis, Hanley & McNeil test including 95% CIs the five biomarkers identified in Response cohort

(A, B, C, D and E, summarised in G) Predictive performance of individual biomarkers, 7934, 15865, 15076, 16129 and 4255 Da were indicated by AUC values of 0.917, 0.865, 0.822, 0.817 and 0.806, respectively. A good discriminating ability of each biomarker was observed with the (F) AUC values > 0.8 and Asymptotic significance < 0.01. AUC value of 1.000 (G) (Asymptotic sig = 0.00 and 95% confidence interval 1.000-1.000) calculated by the combination of the five biomarkers indicating an outstanding predictive ability for the final model.

Abbreviations: Area: AUC (area under the ROC curve), Asymptotic 95% CI: 95% confidence interval, Asymptotic Sig: Hanley & McNeil method, ROC: receiver operating characteristics

Note: The AUC results were considered excellent between 0.9-1, good between 0.8-0.9, fair between 0.7-0.8, poor between 0.6-0.7 and values between 0.5 and 0.6 represent low power of protein to discriminate two groups. Values ≤ 0.5 means no discriminatory power of protein.

6.3.7.2 Spearman Correlations between Three Biomarkers in Response Cohort

Results of Spearman's correlation coefficients ranging from 0.882 to 0.958 ($p = 0.000$) (see Table 6.15) indicated that the expression of 7934 and 15865 Da, 7934 and 16076 Da, 7934 and 16129 Da, 15865 and 16076 Da, 15865 and 16129 Da, 16076 and 16129 Da proteins were highly associated. Four biomarkers; 7934, 15865, 16076 and 16129 Da were highly intercorrelated and also 7934 and 15865 Da proteins were positively correlated with 4255 Da protein at $p < 0.05$ significance level ($R = 0.423$ and $R = 0.424$, respectively). Statistically significant correlation with p-value smaller than 0.05 denotes the similarity between the expression profiles of these biomarkers. No association was found between the expression of 4255 Da protein with 16076 and 16129 Da proteins.

Table 6.15 Spearman correlation for five biomarkers identified in response cohort

			7934q10	15865q10	16076q10	16129q10	4255q10
Spearman's rho	7934q10	Correlation Coefficient	1.000	0.938**	0.882**	0.850**	0.423*
		Sig. (2-tailed)		0.000	0.000	0.000	0.028
		N	27	27	27	27	27
	15865q10	Correlation Coefficient	0.938**	1.000	0.957**	0.931**	0.424*
		Sig. (2-tailed)	0.000		0.000	0.000	0.028
		N	27	27	27	27	27
	16076q10	Correlation Coefficient	0.882**	0.957**	1.000	0.958**	0.372
		Sig. (2-tailed)	0.000	0.000		0.000	0.056
		N	27	27	27	27	27
	16129q10	Correlation Coefficient	0.850**	0.931**	0.958**	1.000	0.318
		Sig. (2-tailed)	0.000	0.000	0.000		0.107
		N	27	27	27	27	27
	4255q10	Correlation Coefficient	0.423*	0.424*	0.372	0.318	1.000
		Sig. (2-tailed)	0.028	0.028	0.056	0.107	
		N	27	27	27	27	27

* Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed), Correlation coefficient (R): 0 = no association between variables, 1 = significant association between variables, N: number of samples

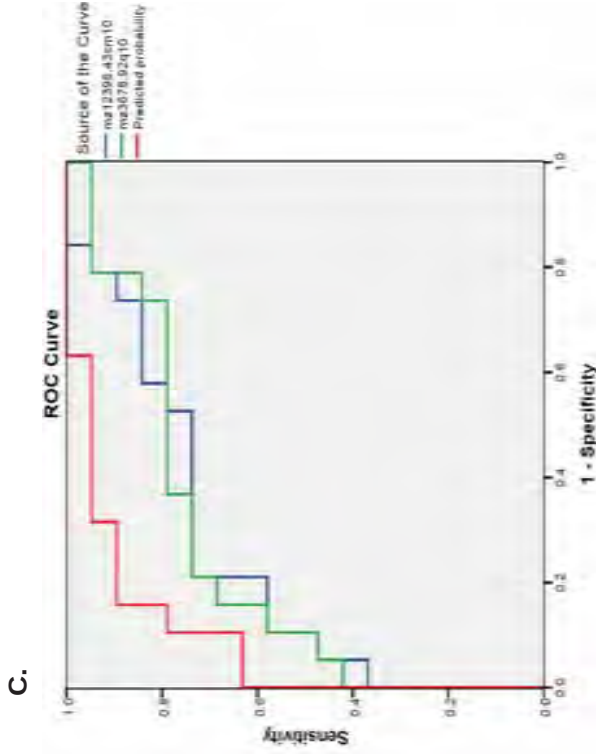
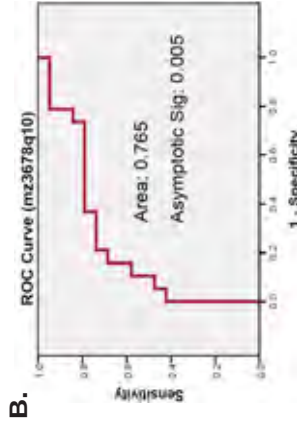
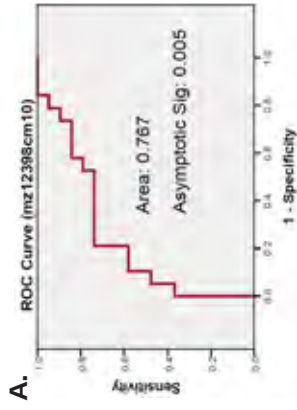
6.3.8 ROC Curve Analysis and Spearman Correlation of Up-regulated Biomarkers in Survival Subgroups

6.3.8.1 ROC Curve Analysis of Selected Two Biomarkers in STS Cohort

Two up-regulated proteins (12398 and 3678 Da) in STS remained in the final BLR biomarker panel. It is important to note that 12398 Da protein was detected within the mass range of 0.3% of 12365 Da biomarker identified in response cohort (see Figure 6.8 in Section 6.3.5). Because these proteins were also collected from the same ProteinChip surface, they were assumed to represent the same protein. Predictive performance of the biomarkers was estimated as AUC values; 0.767 for 12398 Da and 0.765 for 3678 Da proteins as shown in Figure 6.12, A and B. AUC values of both proteins were within the acceptable range with asymptotic significance of 0.05 (Figure 6.12, D). However, broad range of confidence intervals ranging from 0.6 to 0.9 (Figure 6.12, D) displayed a poor predictive precision for each of these biomarkers. Predicating power of the biomarkers in combination (AUC = 0.917, shown in Figure 6.12, C) was shown to discriminate two subgroups of survival with a significant prediction performance (Asymptotic sig. < 0.000). In comparison to individual confidence intervals of the proteins, combination of two biomarkers provided narrower and relatively acceptable confidence intervals (0.823-1.000) (Figure 6.12, D).

6.3.8.2 ROC Curve Analysis of Selected Single Biomarker in LTS Cohort

The BLR analysis for peak selection retained only 15866 Da protein as a predictor of long term survival when overexpressed in patients. The AUC value of 0.731 indicated that the single biomarker had an acceptable predictive ability with asymptotic significance < 0.05 (see Figure 6.12, E). Estimated 95% confidence interval ranging from 0.557 to 0.905 suggested a poor precision of prediction for the biomarker (See Figure 6.12, F).

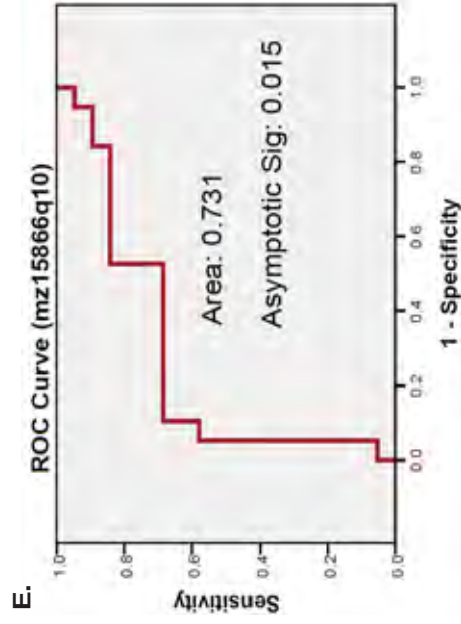


D.

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% CI	
				Lower Bound	Upper Bound
12398cm10	0.767	0.079	0.005	0.613	0.921
3678q10	0.765	0.082	0.005	0.604	0.926
Predicted probability	0.917	0.045	0.000	0.823	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5



F.

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% CI	
				Lower Bound	Upper Bound
15866q10	0.731	0.089	0.015	0.557	0.905

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 6.12 ROC curve analysis, Hanley & McNeil test including 95% CIs the two biomarkers identified in survival cohort

A fair predictive performance of the up-regulated proteins in STS cohort was represented by AUC values of 0.767 (confidence interval, 0.613-0.921) and 0.765 (confidence interval, 0.604-0.926) for biomarkers (A) 12398 Da and (B) 3678 Da, respectively. An AUC value of (C) 0.917 suggesting relatively high predictive ability was calculated by the combination of two biomarkers (D) combination of the biomarkers also revealed an acceptable confidence interval range with Asymptotic sig < 0.000 and 95% confidence interval, 0.823-1.000.

(E) BLR analysis of up-regulated proteins in LTS revealed 15866 Da protein as a predictor of long term survival with an AUC value of 0.731 (Asymptotic sig. < 0.05) indicating an acceptable prediction performance. (F) However, 95% confidence interval ranging from 0.557 to 0.905 suggested a poor precision of prediction for the selected biomarker.

Abbreviations: Area: AUC (area under the ROC curve), Asymptotic 95% CI: 95% confidence interval, Asymptotic Sig: Hanley & McNeil method, ROC: receiver operating characteristics

Note: The AUC results were considered excellent between 0.9-1, good between 0.8-0.9, fair between 0.7-0.8, poor between 0.6-0.7 and values between 0.5 and 0.6 represent low power of protein to discriminate two groups. Values ≤ 0.5 means no discriminatory power of protein.

6.3.8.3 Spearman Correlations between Selected Two Biomarkers in STS Cohort

Calculation of Spearman's correlation coefficient ($R = 0.124$) (see Table 6.16) revealed a weak positive relationship between 12398 and 3678 Da proteins. No significant ($p > 0.05$) association was found between expression profiles of the two biomarkers up-regulated in STS cohort.

Table 6.16 Spearman correlation for two biomarkers identified in survival cohort

			12398cm10	3678q10
Spearman's rho	12398cm10	Correlation Coefficient	1.000	0.124
		Sig. (2-tailed)		0.458
		N	38	38
	3678q10	Correlation Coefficient	0.124	1.000
		Sig. (2-tailed)	0.458	
		N	38	38

* Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed), Correlation coefficient (R): 0 = no association between variables, 1 = significant association between variables, N: number of samples

6.3.9 Protein Purification and Identification

6.3.9.1 Semi-purification of Protein Samples

Three biomarkers up-regulated in non-responders were selected for further purification and identification. Presence or absence of proteins of interest in the semi-purified fractions was confirmed on NP20 ProteinChips. Figure 6.13 shows representative protein profiles from 7.2 to 13 kDa mass range analysed by SELDI-TOF MS. Fractionation of crude protein samples resulted in elution of expected proteins (10.2, 10.8 and 12.3 kDa) abundantly in the 50% ACN buffer.

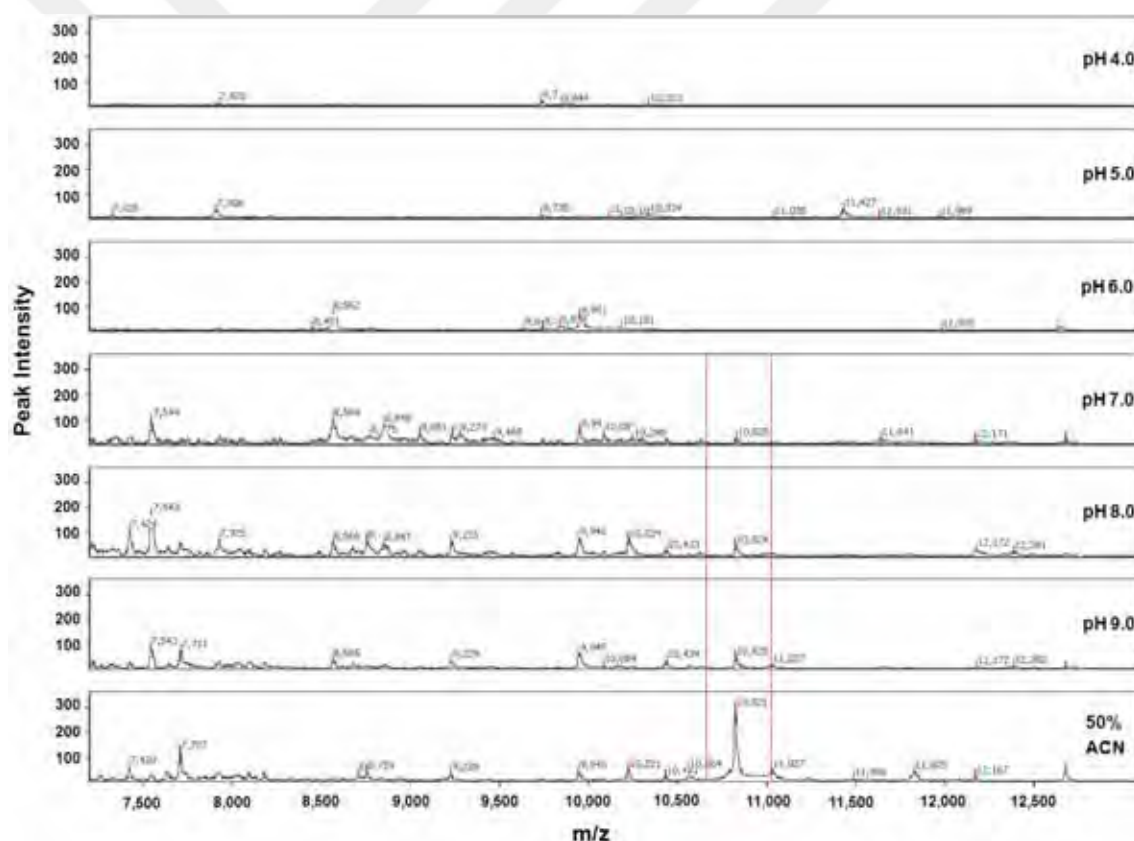


Figure 6.13 Representative spectra for the semi-purification of proteins using Mustang S Filter Plate

SELDI-TOF MS protein profiles from 7.2 to 13 kDa illustrates molecular mass on x-axis and peak intensity on y-axis. Protein peaks in red box indicated presence of 10.8 kDa biomarker in semi-purified fractions with buffers; pH 7.0, 8.0, 9.0 but mainly in 50% ACN buffer.

6.3.9.2 SDS-PAGE Separation of Semi-purified Protein Fractions

After the BLR analysis of significant peaks in response and survival groups, up-regulated peaks in non-responders at m/z 10,247, 12,361 and 10,850 were identified by the further purification of glioma tissue extracts using an ion-exchange separation protocol. Figure 6.14, A, B and C illustrate the semi-purified fractions monitored on NP20 chips in order to identify the fractions containing expected mass peaks.

Inadequate amounts of protein were isolated when semi-purified samples further separated by RP-HPLC. Total protein concentrations of RP-HPLC fractions containing proteins of interest were measured by Bio-Rad protein assay. Detected concentrations were all below 10 $\mu\text{g}/\text{fraction}$ which was the detection limit of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). RP-HPLC fractions in liquid form (lyophilised and reconstituted in PBS) and also SDS-PAGE separated (SYPRO Ruby stained) barely visible protein bands, however these were sent for the protein identification. Peptide matches on database with the Mascot search engine did not reveal peptides with expected molecular masses. Therefore for the subsequent analysis of protein identification, semi-purified fractions containing candidate biomarker peaks were resolved directly by SDS-PAGE before sending for the LC-MS/MS analysis.

Figure 6.14, D, E and F show further resolution of identified fractions performed on 12% and 20% SDS-PAGE. Following separation of the samples, bands were visualised by SYPRO Ruby protein stain. The bands had a mass corresponding to m/z ratio of 10.2, 12.3 and 10.8 kDa which were indicated by arrows in the second lane of the gel panels in Figure 6.14, D, E and F, were excised from the gel and send for the protein identification by LC-MS/MS.

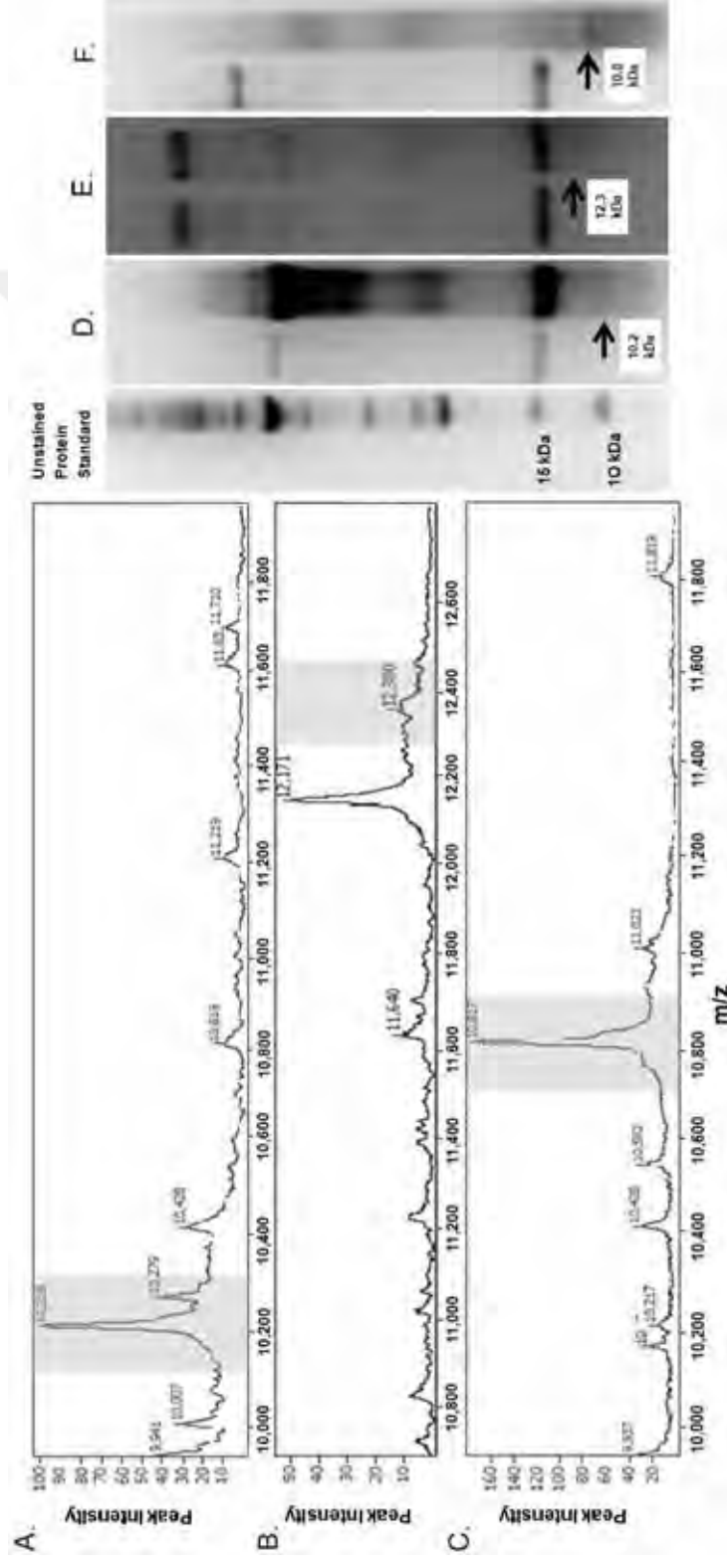


Figure 6.14 Analysis of semi-purified fractions on NP20 arrays

SELDI-TOF mass spectra of the glioma samples after fractionation. Peak intensities are shown on y-axis and molecular masses on x-axis. Peaks of interests were highlighted with light grey background at **(A)** 10.2, **(B)** 12.3 and **(C)** 10.8 kDa. Fractions in 50% ACN were lyophilised and reconstituted in PBS. Concentrated samples were subjected to SDS-PAGE, arrows indicate bands containing **(D)** 10.2, **(E)** 12.3 and **(F)** 10.8 kDa proteins. Bands in the relevant mass range were excised and sent for the MS/MS analysis.

Note: D and E, electrophoresis of samples on 12% NuPAGE Novex Gel (Invitrogen, Carlsbad, CA, USA). F, electrophoresis of samples on 4-20% Mini-PROTEAN TGX (Bio-Rad Laboratories, Hercules, CA, USA).

6.3.9.3 Identification of Proteins

Mascot search reports of protein tryptic digests from semi-purified and SDS-PAGE separated fractions identified specific sequences of the three selected biomarkers. LC-MS/MS results revealed the identity of the 10.2, 12.3 and 10.8 kDa proteins to be Alpha Defensin-3 Preproprotein (DEFA3), Macrophage Inhibitory Factor (MIF) and Calgranulin A (S100A8), respectively. The theoretical masses of the proteins obtained from the Uniprot database '<http://www.uniprot.org/uniprot>' was 10245 for DEFA3, 12476 for MIF and 10835 Da for S100A8. Proteins were identified when a significant ($p < 0.05$) MS/MS ion score more than 42 was obtained and a sequence coverage of at least 10% was achieved. The identified proteins (DEFA3 = 150, MIF = 65, S100A8 = 381) all had significant ion scores. The identities of the proteins were confirmed by detection of sequences highlighted in red in the full protein sequence shown in Table 6.17. 22%, 10% and 57% of the full sequences of the proteins were covered by the identification of 21 amino acids for DEFA3, 11 amino acids for MIF and 53 amino acids [including N-terminal with methionine (M)] for S100A8, respectively. Processes prior to protein identification might cause posttranslational modifications. An alkylated peptide containing carbamidomethylated cysteine (C) was identified in tryptic digests of MIF protein.

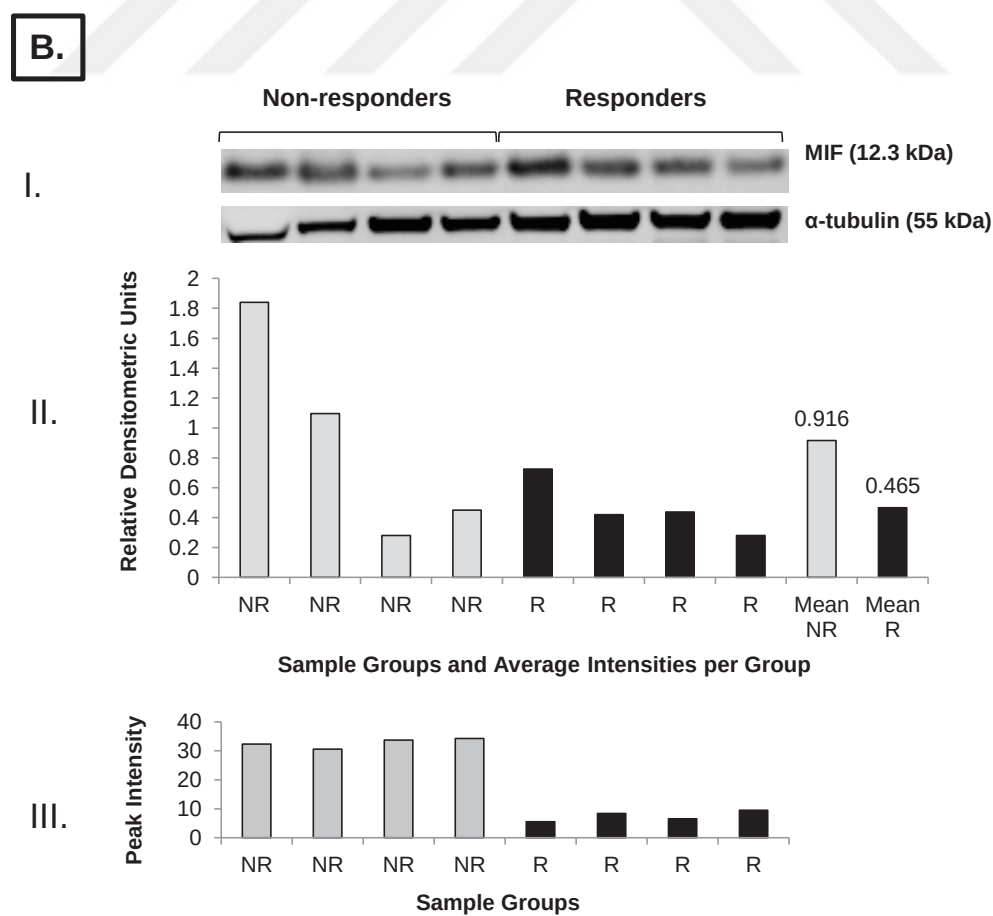
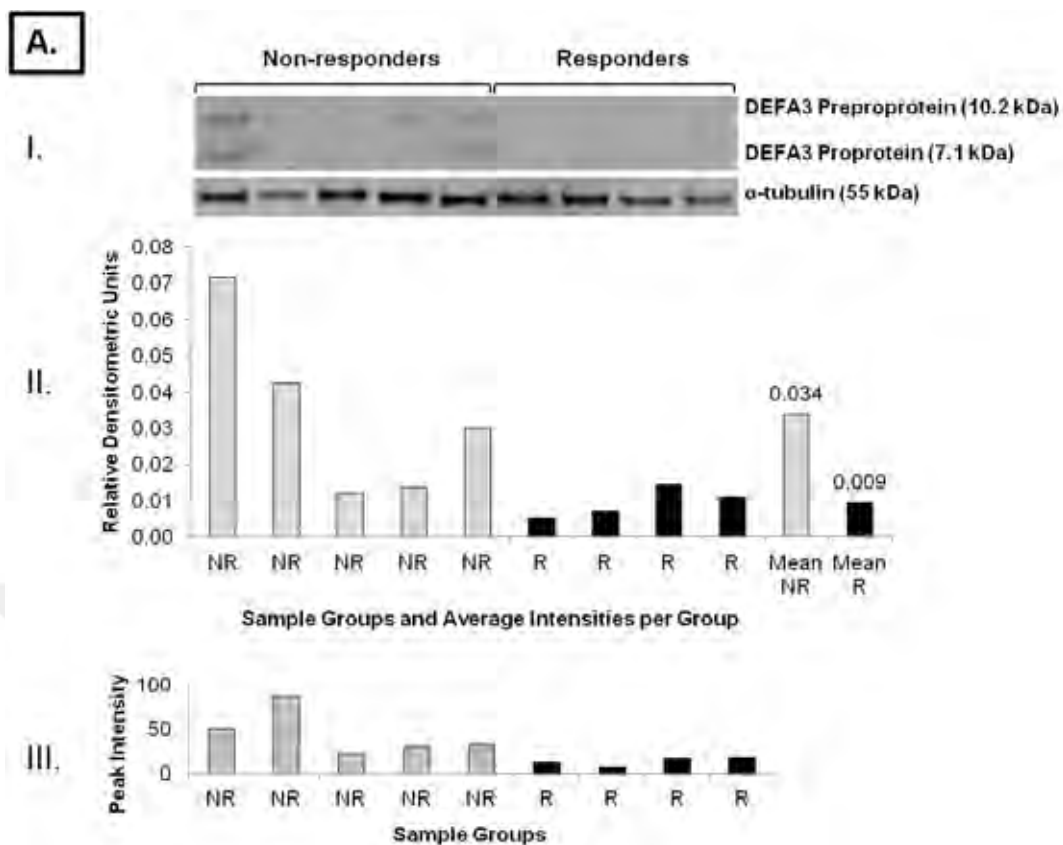
Table 6.17 Summary of Mascot search results for identified proteins

Protein Name	m/z UniProt ^a	m/z Observed Cluster	Peptides Identified ^e	MS/MS Ion Scores ^f	Sequence ^g	Sequence Coverage
DEFA3	10245 ^b	10247	R. IPACIAGER .R R.YGTCTIYQGR.L R. IPACIAGERR .Y R.RYGTCTIYQGR.L Total 150	64 40 25 21 Total 150	10 MRTLAITLAAI LLVALQAQE PLQARADEVA AAPEQIAADI PEVVVSLAWD ESLAPKHPGS 70 RKNMDCYC RI PACI AGER RY GTCTIYQGR LW AFCC (94 amino acids)	22%
MIF	12476 ^c	12361	K.LLC* G LLAER.L Total 65	65 Total 65	10 MPMFIVNTNV PRASVPDGF L SELTQQLAQA TGKPPQYIAV HVPDQQLMAF GGSSEPCALC 70 SLHSIGKIGG AQNRSYS KLL C G LLAERLRI SPDRVYINYY DMNAANVGWN NSTFA (115 amino acids)	10%
S100A8	10835 ^d	10850	K.GADVWFK.E -..MLTELEK.A K.GNFAVYR.D K.ALNSIIDVYHK.Y K.LLETECPQYIR.K K.GNFAVYRDDLK.K K.LLETECPQYIRK.K K.LLETECPQYIR.K Total 381	37 27 35 55 86 34 51 59 Total 381	10 ML TELEKALN SIIDVYHKYS LIKGNFHAVY RDDLK KLLET ECPQYIR KKG ADVWFKELDI 70 NTDGAVNFOE FLILVIKMGV AAHKKSHEES HKE (93 amino acids)	57%

^a Masses were obtained from the uniprot database “<http://www.uniprot.org/uniprot>”; ^b Uniprot accession number, P59666; ^c Uniprot accession number, P14174; ^d Uniprot accession number, P05109; ^e amino acid sequence of peptides identified from tryptic digests, C* denotes carbamidomethyl, MASCOT search parameters included carbamidomethylation of cysteine as a variable modification; ^f MS/MS ion scores obtained from MASCOT database, individual ion scores > 42 indicate identity or extensive homology (p < 0.05); ^g Highlighted letters in the full sequence show the covered sequence by the identified peptides.

6.3.10 Validation of Identified Biomarkers in Response and Survival Patient Cohorts by Immunoblotting

Immunoblotting with S100A8, DEFA-3 and MIF antibodies were performed to confirm association between expression levels of proteins and response outcome using the protein samples of the same patient cohort. Based on peak intensities from SELDI-TOF MS, patients with high protein peaks from non-responders and patients with low protein peaks from responders were selected as a small subset of the patient cohort. Differential expression results obtained by SELDI-TOF MS were confirmed using antibodies specific for DEFA3, MIF and S100A8. Overexpression in non-responders was confirmed for all of the biomarkers using Western blot analysis (see Figure 6.15). Probing with the DEFA3 antibody bound to both pre-proprotein (10.2 kDa) and proprotein (7.1 kDa) form of the DEFA3 protein resulting in the presence of two bands corresponding to proteins with molecular masses of 10.2 and 7.1 kDa. Figure 6.15, AI and AII. In Figure 6.15, BI and BII, high levels of MIF as indicated by the single band at 12.3 kDa were detected in the non-responders. Expression of S100A8 protein was confirmed with the protein band detected at 10.8 kDa exhibiting the highest expression fold change ratio in non-responders compared to responders (see figure 6.15, CI and CII). Even though some variations between results from immunoblotting and SELDI-TOF MS profiling were noted, overall expression patterns were consistent. Verification of MIF differential expression was also evidenced in survival cohort by immunoblotting in order to confirm expression profiles of 12398 Da biomarker found overexpressed in STS cohort (see Figure 6.16, A and B). Analysis of bands at 12.3 kDa generated consistent trends of protein expression in regard with peak intensities identified by SELDI-TOF MS (see Figure 6.16, C).



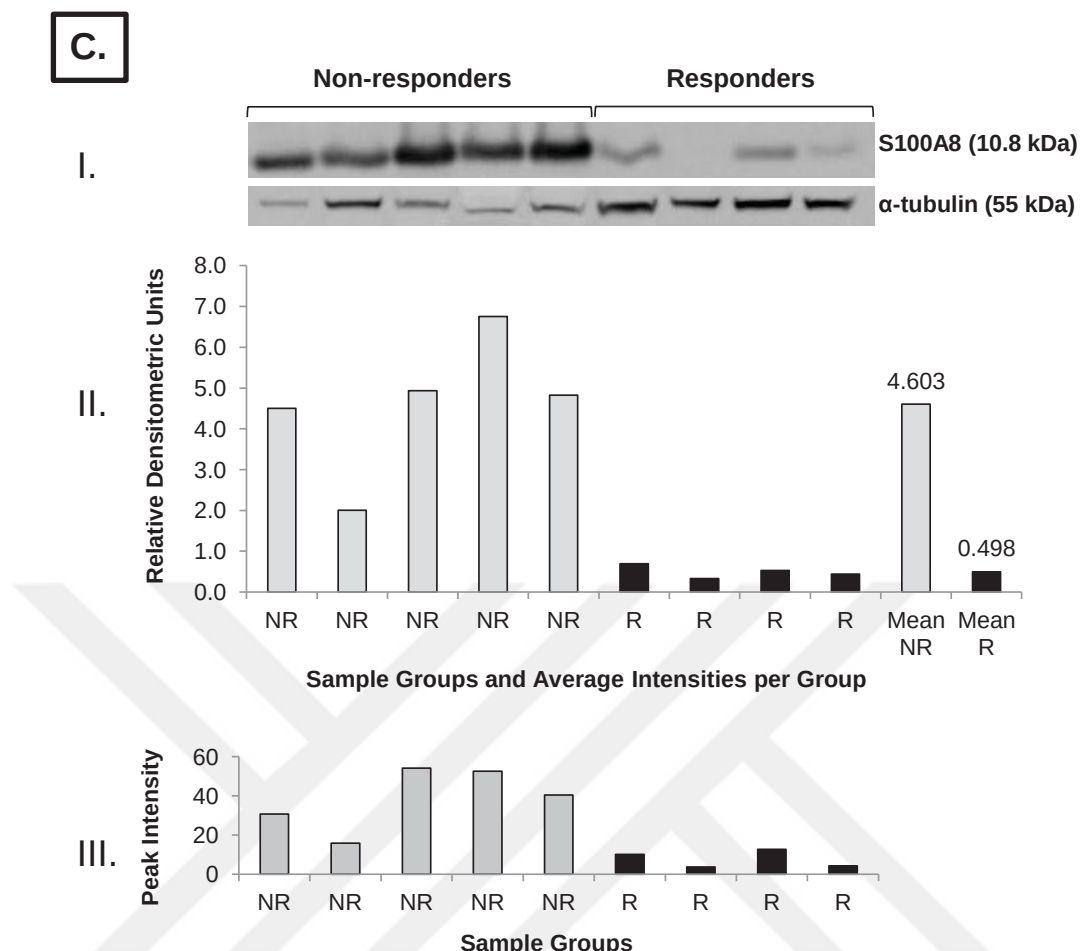


Figure 6.15 Validation of the three identified proteins DEFA3 (A), MIF (B), and S100A8 (C) by western blotting in response cohort

For the separation of proteins on the gel, amount of the protein loaded in each lane was 30 µg. Immunoblotting of proteins was performed using **(A)** Alpha Defensin (1+2+3) polyclonal goat antibody at 1:160, **(B)** MIF polyclonal goat antibody at 1:200, **(C)** S100A8 polyclonal sheep antibody at 1:200 and **(A, B and C)** α-tubulin monoclonal mouse antibody as a loading control at 1:2000 dilutions. **(AI, BI and CI)** Representative immunoblotting of proteins. **(AII, BII and CII)** Densitometric analysis of bands calculated relative to α-tubulin. The last two bars at right demonstrating the average protein intensities for groups. **(AIII, BIII and CII)** The samples selected on the basis of high or low intensity of peaks detected by SELDI-TOF MS. All figures are the representative of the experiments repeated three times.

Abbreviations: DEFA3: alpha defensin 3, MIF: macrophage inhibitory factor, NR: non-responder, R: responders, S100A8: Calgranulin A

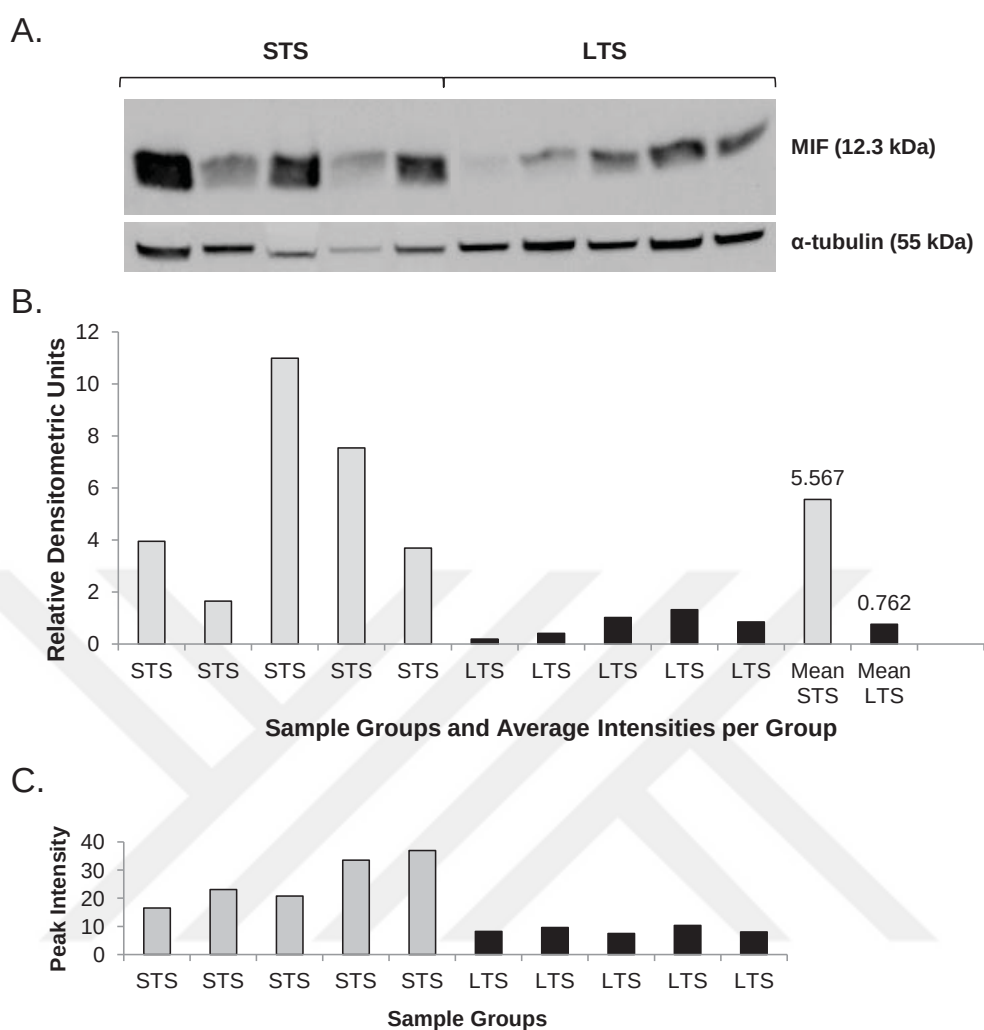


Figure 6.16 Validation of MIF in survival cohort by immunoblotting

(A) For the separation of proteins on the gel, 30 μ g of the protein was loaded in each lane. Immunoblotting of the samples was performed using MIF polyclonal goat antibody at 1:200 and α -tubulin monoclonal mouse antibody as a loading control at 1:2000 dilutions. Representative immunoblotting of proteins. **(B)** Densitometric analysis of bands relative to α -tubulin. The last two bars at right demonstrating the average protein intensities for groups. **(C)** SELDI-TOF MS intensities of MIF protein corresponding 12398 kDa protein. The figure is the representative of the experiments repeated three times.

Abbreviations: LTS: long term survivors, MIF: macrophage inhibitory factor, STS: short term survivors

6.3.11 Expression of Identified Biomarkers in GNS Cultures and Glioma Cell Lines

Immunoblotting was performed using cell lysates and cell culture supernatants (conditioned medium) of GNS and glioma cell lines. Non-responder patient sample shown in Figure 6.17 and Figure 6.18 was served as a positive control of DEFA3, MIF and S100A8 protein expression. The presence of a defensin isoform with an approximate molecular weight of 14 kDa was suggested to be a potential dimer formation of prodefensins (7.1 kDa) in cell lysate (see Figure 6.17). No expression of DEFA3 was detected in conditioned medium from GNS and glioma cell lines (see Figure 6.18). Expression of MIF protein in cell lysates was also detected in the absence of complete tumour microenvironment (see Figure 6.17). However no detectable signal was observed for MIF in conditioned medium from GNS and glioma cell lines (see Figure 6.18). Expression of S100A8 was detected neither in the cell lysates nor conditioned medium but only in patient tumour samples as shown in Figure 6.17 and 6.18. Presence of MIF, a different isoform of DEFA3 and lack of S100A8 expression *in vitro* was also confirmed using BAH1 GNS cell lysates (shown in Figure 6.18).

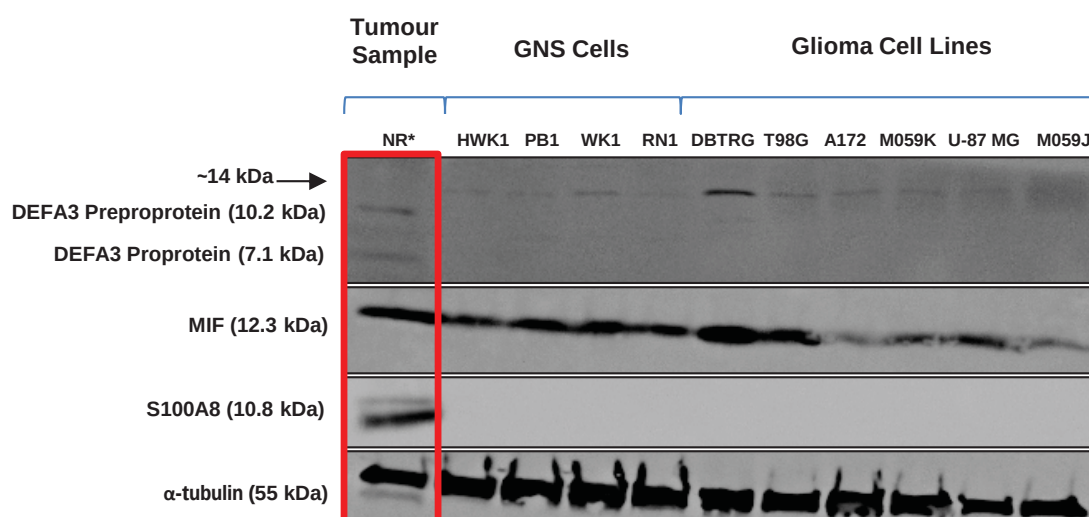


Figure 6.17 Expression of the three biomarkers in cell lysates of GNS and glioma cell lines

Protein expression in positive control was highlighted by a red frame. *: non-responder patient tumour sample used as positive control

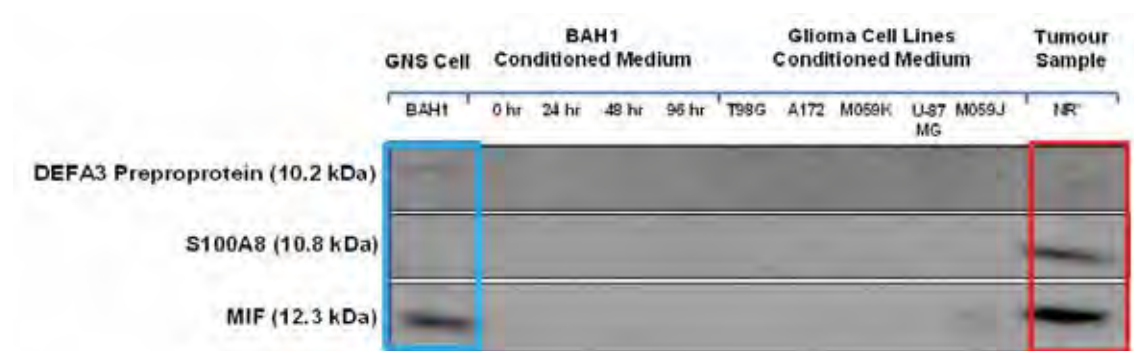


Figure 6.18 Expression of three biomarkers in BAH1 GNS cell lysate and conditioned medium of BAH1 GNS cells and glioma cell lines

Protein expression in positive control and BAH1 GNS cell lysate were highlighted by red and blue frames, respectively. *: non-responder patient tumour sample used as positive control

6.3.12 Immunohistochemical Validation of Identified Biomarkers

The expression of DEFA, S100A8 and MIF proteins in tumour sections of patients with newly diagnosed glioblastoma (validation cohort) were assessed by IHC. Unfortunately due to the delay of obtaining clinical samples (> than 4 months after initial request, the analysis and scoring of tumour sections for DEFA3 and S100A8 are still in progress. Therefore, the localisation of the proteins and positive and intermediate and negative representative images of the staining is provided in this thesis. Figure 6.19 shows human tonsil (FFPE), spleen (FFPE) and breast cancer (FFPE) tissue sections stained for DEFA3, S100A8 and MIF, respectively, as positive controls.

Immunohistochemical staining of tumour sections for DEFA3 showed scattered DEFA3-positive cells which were specific to neutrophils (confirmed by pathologist) (see Figure 6.20 and 6.22).

S100A8 staining of the glioma sections demonstrated infiltrative S100A8-positive inflammatory cells which were predominantly identified as neutrophils and macrophages (see Figure 6.21 and 6.22). S100A8-positive cells exhibited a scattered distribution throughout the tumour section which was found to be a similar distribution pattern of DEFA3-positive cells.

A representative low magnification image demonstrating distribution of the cells stained for MIF is shown in Figure 6.22. MIF IHC showed intense tumour cell staining with variable level of cytoplasmic localisation of MIF expression (see Figure 6.23 and 6.24). Diffuse staining of MIF-positive cells was detected throughout the tumour section with some areas of more intense staining that appeared as patchy distribution of MIF-positive cells. IHC for MIF also revealed intense staining in palisading area around the areas of necrosis.

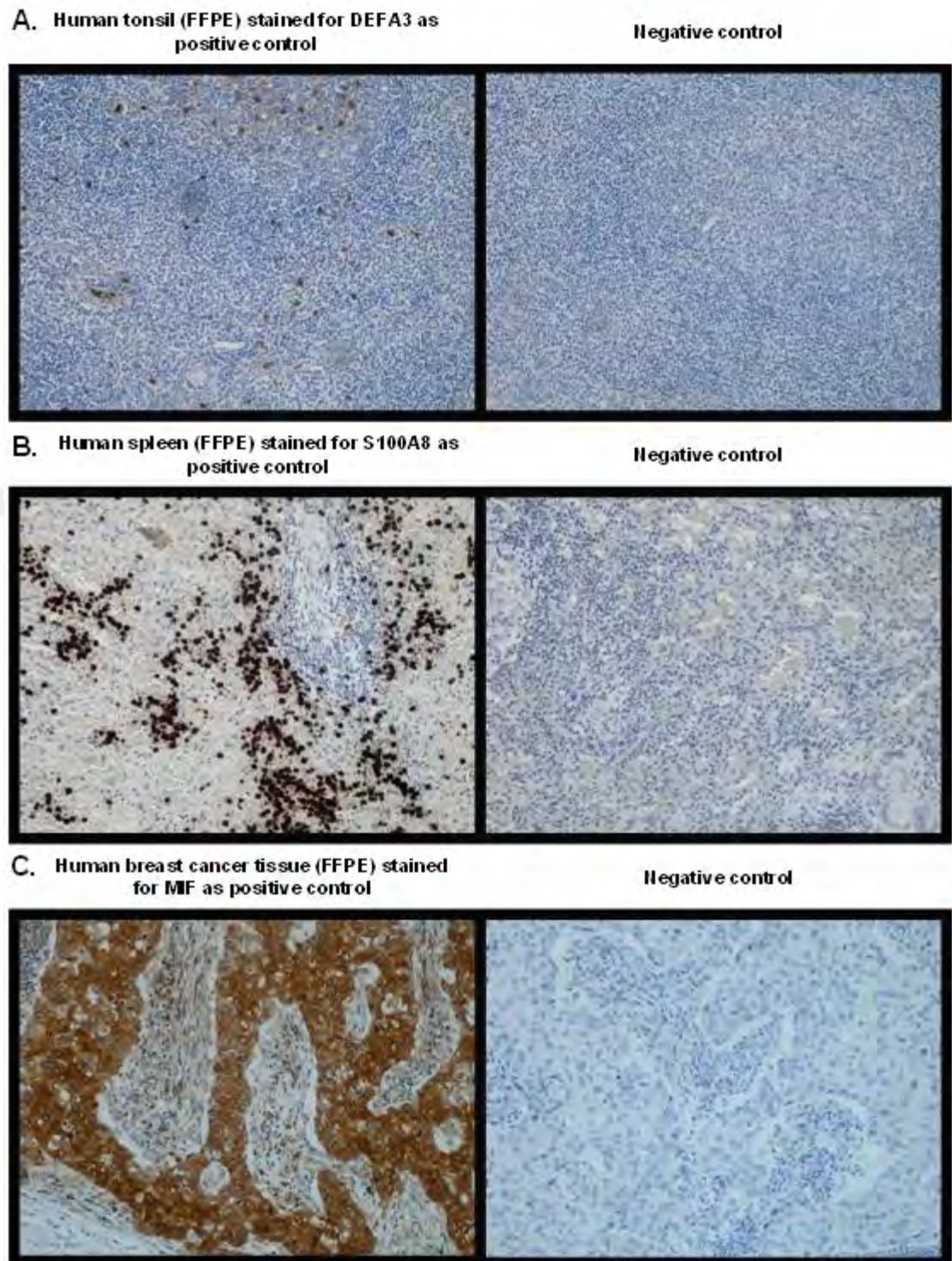


Figure 6.19 Representative images of human tissues used as positive controls

Immunohistochemical staining of human tonsil for **(A)** DEFA3, human spleen for **(B)** S100A8 and human breast cancer tissue for **(C)** MIF, (magnification 20X)

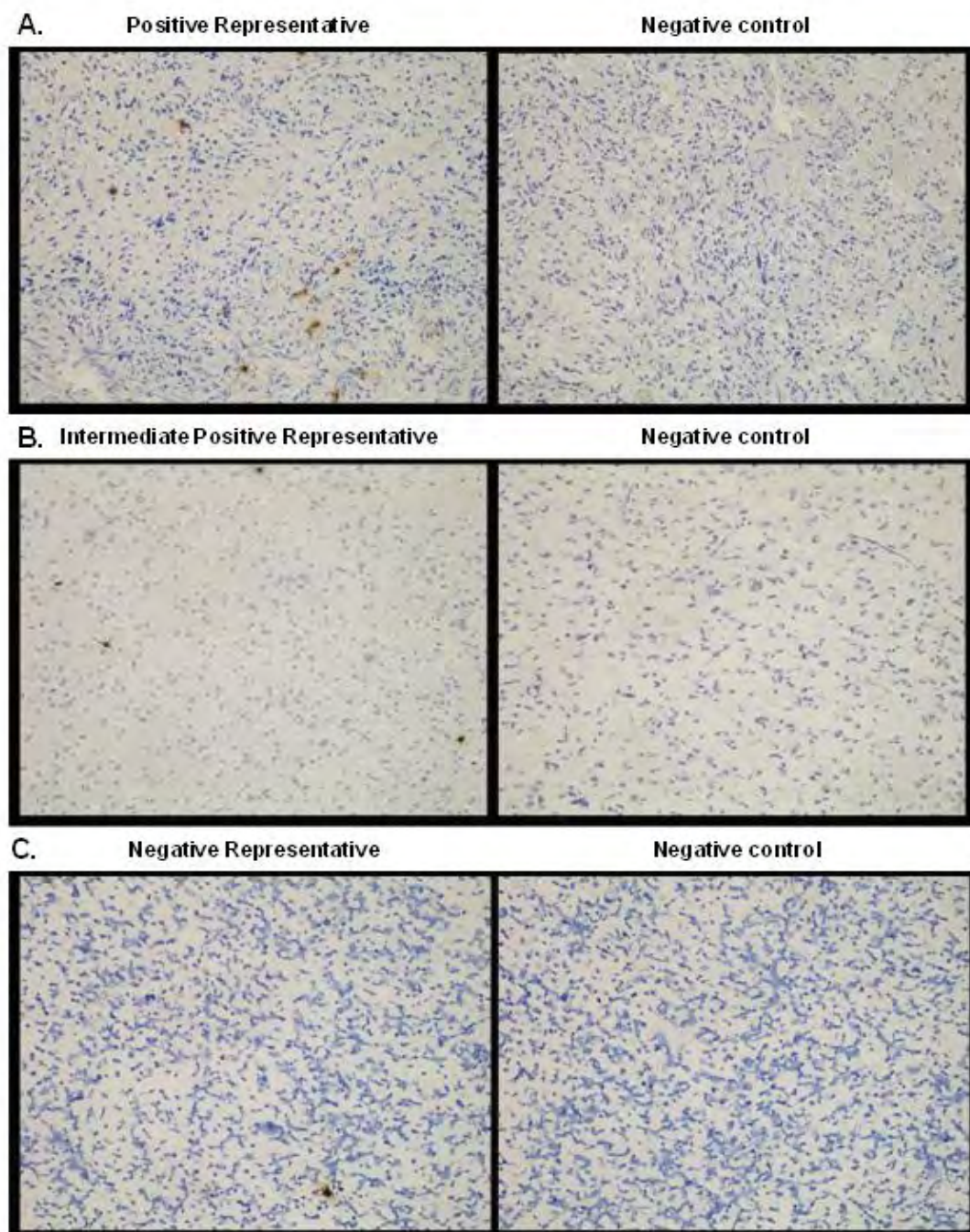


Figure 6.20 Immunohistochemical analysis of human DEFA3 expression in tumour samples from the patients with newly diagnosed glioblastoma

DEFA3 protein expression detected by IHC using a specific monoclonal mouse antibody. The immunoreactivity for DEFA3 in A, B and C demonstrates neutrophil infiltration on tumour sections. DEFA3-positive cells show also immunoreactive material around them. **(A)** Positive, **(B)** intermediate and **(C)** negative representative images of DEFA3 stained sections show staining of the neutrophilic cells (magnification, 20X).

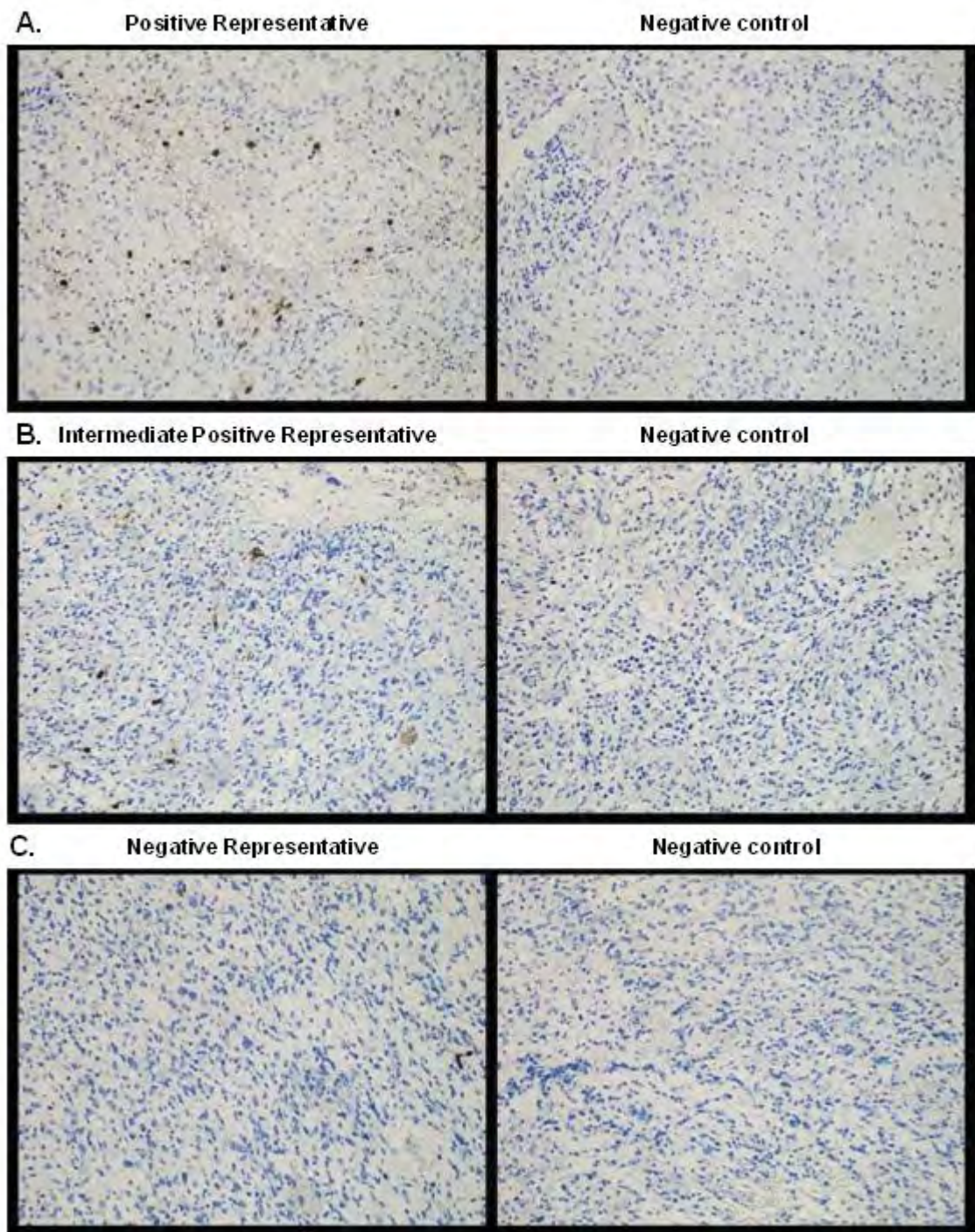


Figure 6.21 Immunohistochemical analysis of human S100A8 expression in tumour samples from the patients with newly diagnosed glioblastoma

S100A8 protein expression detected by IHC using a specific polyclonal rabbit antibody. **(A)** Positive, **(B)** moderate and **(C)** negative representative images are indicated with variable number of S100A8-positive cells, (magnification 20X).

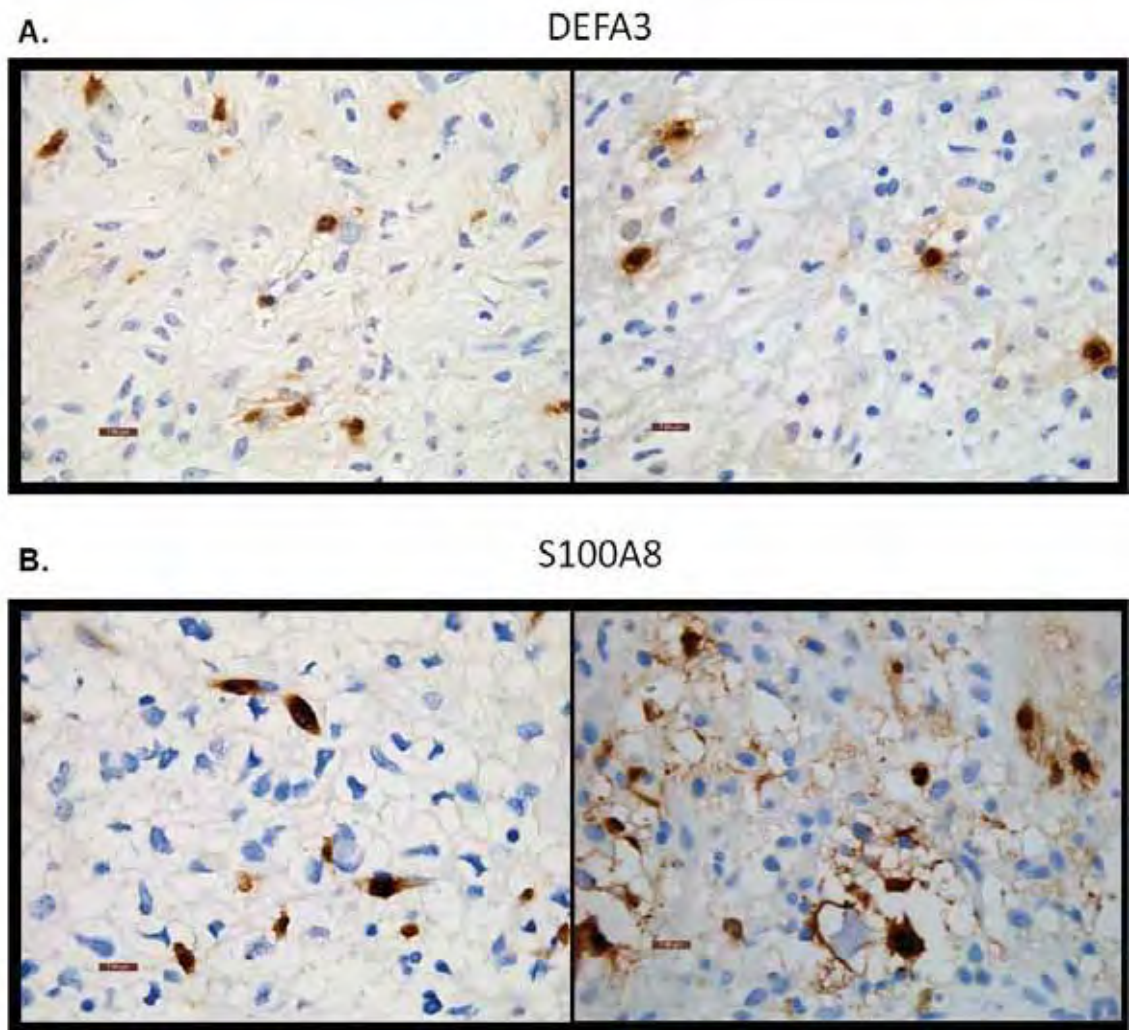


Figure 6.22 Representative immunohistochemical staining for DEFA3 and S100A8 (Higher Magnification-63X)

Representative images for IHC staining is shown in **(A)** DEFA3 **(B)** S100A8 (magnification 63X).

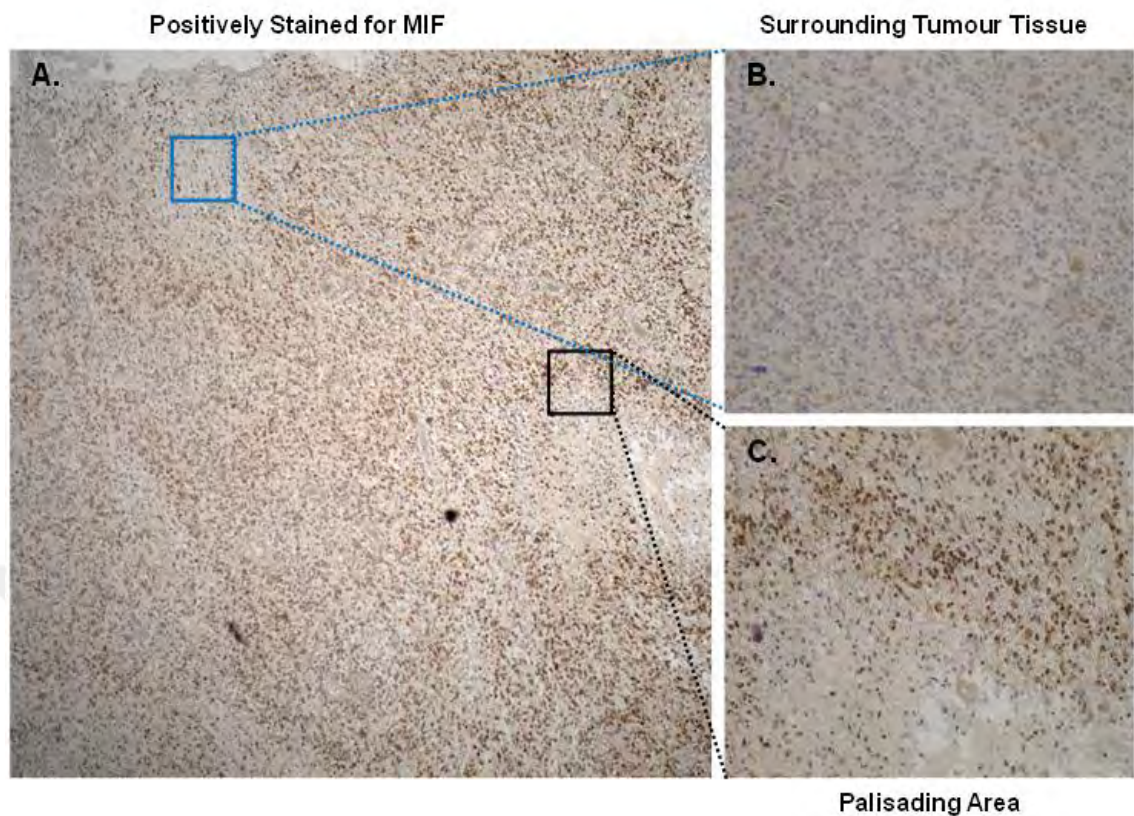


Figure 6.23 Representative immunohistochemical staining for MIF showing distribution of MIF-positive cells

MIF protein expression detected by IHC using a specific monoclonal mouse antibody. (A) Representative low magnification image, (magnification 4X) The micrographs in (B) and (C) are five-fold magnifications of the designated areas indicated by a blue and black square in A, (magnification 20X). While B demonstrates MIF expression in surrounding tumour tissue, MIF expression in palisading area of the tumour around the necrosis is shown in C.



Figure 6.24 Immunohistochemical analysis of human MIF expression in tumour samples from the patients with newly diagnosed glioblastoma

MIF protein expression detected by IHC using a specific monoclonal mouse antibody. A representative image from a **(A)** positively, **(B)** moderately and **(C)** negatively stained tissue is shown with negative controls of each (magnification 20X).

6.4 DISCUSSION

The primary aim of this study was to explore variations in the expression patterns of small molecular weight proteins in glioma patient subgroups using SELDI-TOF MS technology. SELDI-TOF MS, ProteinChip arrays have been efficiently employed for biomarker identification in several cancer types predominantly using serum samples (Combaret et al., 2005; Lei et al., 2011; Zhang et al., 2006). In the present study, four ProteinChip arrays used for the analysis of protein samples were namely, H50, Q10, CM10 and IMAC. Classical chromatographic separation of protein samples on different array surfaces involved reverse phase (H50), ion exchange (Q10 and CM10) and immobilised metal affinity capture (IMAC) techniques based on chemical interactions between biomolecules and chemically modified surfaces (Merchant and Weinberger, 2000). Binding buffer conditions were optimised for Q10 and CM10 arrays and reproducible results were obtained by the application of buffers with pH 8.5 and pH 4.0 respectively for each array. Similar pH conditions for binding buffers have also been reported in an optimisation study conducted with urine samples of 8 healthy subjects using SELDI-TOF MS (Papale et al., 2007). At low pH, proteins within the sample are positively charged that favour their binding to a weak cationic exchanger, CM10 array surface. High pH of binding buffer used for Q10 array employs quaternised ammonium groups as a strong anion exchanger to increase binding of the negatively charged proteins on the array surface. In H50 array, hydrophobicity of the surface is determined depending on the concentration of organic solvent used in the binding buffer (Papale et al., 2007). 10% ACN-0.1% TFA was used to selectively capture proteins which were more hydrophobic than the binding buffer (Scarlett et al., 2006). Optimisation of the binding buffer with TFA concentrations from 0.1 to 0.5% decreased the number of peaks detected in the mass range of 2.5-30 kDa. However acetonitrile concentration had

no effect on results (Panicker et al., 2009b). IMAC array activated with copper was used to detect overall protein profile of samples (Felix et al., 2011). Immobilised Cu^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} ions interact with the general amino acid residues (imidazolyl, thiol and indolyl functional groups) on protein surfaces, while phosphorylated proteins can be specifically selected by Fe^{3+} coupled IMAC arrays (Ueda et al., 2003).

Inter-chip variabilities of ProteinChip arrays were calculated from the peak intensities of insulin standard as CV%; 4.9, 16.42, 11.97 and 16.3 for H50, Q10, CM10 and IMAC, respectively. These inter-chip variability values were under 20% and in agreement with the previously reported CV% in proteomics studies (Panicker et al., 2009b; Ranganathan et al., 2005). Normalisation of protein intensities to the average TIC gave rise to very high or low NF deviated by more than two standard deviations from the mean NF resulted in elimination of 7 spectra from response and 10 spectra from survival cohort before analysis. Elimination of deviation-affected spectra using this normalisation methodology were also used by other proteomics studies to correct systematic variation between spectra (Grus et al., 2005; Miyamae et al., 2005; Sauve and Speed, 2004; van Winden et al., 2009). In a few cases, very poor protein profile was observed in the eliminated spectra when compared to spectra with normal NF, possibly due to ion suppression caused by buffer remnants on the chip surface (Engwegen et al., 2007).

Using four different ProteinChip arrays, we initially identified 225, 88, 198 and 123 common peaks in 54 protein profiles from response cohort and 68, 81, 76 and 43 common peaks in 76 protein profiles from survival cohort using H50, Q10, CM10 and IMAC arrays, respectively. Total number of 44 peak clusters for response and 39 protein peaks for survival cohort was significantly different between patient groups. In

glioma proteomics, only two studies to date have reported the use of more than two types of ProteinChip arrays in combination (Bouamrani et al., 2006b; Zhang et al., 2007b). Even though some peaks were identified by more than one array type, each chemically modified surface mainly revealed different sets of proteins. In this study, combination of the data collected by H50, Q10, CM10 and IMAC ProteinChip arrays provided extensive proteomic information for each glioma specimen. Kristina and colleagues suggested that ProteinChip array combination involving IMAC, H50 and NP20 (alkaline wash) was the most efficient surface types which revealed 92% of all detected peaks (Kristina et al., 2009). As previously reported by others, a data fusion methodology was used to further evaluate the potential of protein peaks in discriminating between groups (Forshed et al., 2008; Vlahou et al., 2003). All data was initially processed using Mann Whitney-U test to calculate significance of peaks from the data set collected by each chip surface. Following Mann Whitney-U analysis, data fusion was conducted in a hierarchical way by concentrating data from separate ProteinChip arrays. Therefore, the most significant protein peaks were selected from all data and analysed by BLR to generate powerful biomarker panels for a prediction model.

Results of the backward elimination by BLR analysis generated the predictor models by 100% sensitivity and specificity (AUC = 1.000) in combination of three and five up-regulated biomarkers in non-responder and responder patient groups, respectively. Two up-regulated biomarkers were revealed by BLR analysis in STS group and predictability of the final model was powerful with AUC value, 0.917 (sensitivity and specificity, 84.2%). Whereas, only a single biomarker suggesting an acceptable predictive ability with AUC value of 0.731 (sensitivity = 68.4% and specificity = 84.2%) was determined in LTS group. Combinations of biomarkers resulted in an improved predictive power

which provided superior information to any individual marker and showed robust discrimination between two patient groups. Other SELDI-TOF MS studies have also developed multiple biomarker panels to increase predictive ability of individual markers for ovarian and pancreatic cancers (Kozak et al., 2003a; Xue et al., 2010). Zhang and co-workers suggested a panel of 7 serum biomarkers to significantly discriminate astrocytoma (WHO Grade I, II, III and IV) from normal controls with 84.6% sensitivity and 86.4% specificity. Two of these markers, m/z 7567 and 15115 with unknown protein identity, were found to be expressed significantly higher in low grade astrocytoma (WHO Grade I-II) compared with high grade astrocytoma (WHO Grade III-IV) (Zhang et al., 2007b). In the selection of an ideal biomarker, features such as high specificity and sensitivity are required in order to prevent false-positive and false-negative detections. Higher discriminatory ability of a test is critically important in reliable classification of patient groups (Allen and Felker, 2010).

Following BLR analysis, marker panels were prioritised for further identification and validation according to complementary predictive performance based on sensitivity and specificity of markers (Williams et al., 2006). Two panels of multi-marker combinations (three markers up-regulated in non-responders and five markers up-regulated in responders) were both equally the most effective in discriminating between response groups. However, three-biomarker model was selected for its potential feasibility as a relatively small biomarker set with high effectiveness (Baker, 2005).

In the present work, three proteins in our biomarker panel were identified as Alpha Defensin-3 Preproprotein (DEFA3) (10.2 kDa), Macrophage Inhibitory Factor (MIF) (12.3 kDa) and Calgranulin A (S100A8) (10.8 kDa) by LC-MS/MS using MASCOT

search database. The 12.3 kDa MIF protein was up-regulated both in the non-responder and STS cohorts. However, because of the technical differences in the purification and identification stages of the biomarkers < 4 kDa, identification of 3.6 kDa protein up-regulated in STS could not be achieved.

Difference between the expected and observed molecular masses of identified proteins was only observed in the MIF protein. Alterations in molecular weight of peptides can occur as a result of post-translational modifications (Mann and Jensen, 2003). Although a precursor form of 115 amino acid (aa) long MIF protein has a molecular mass of approximately 12.5 kDa, cleavage of the initiating methionine yields a mature protein of 114 aa with a molecular mass of 12.3 kDa with an N-terminal proline residue (Swope et al., 1998; Thorat et al., 2010). However, carbamidomethylation modification of the cysteine residue observed in the peptide fragment of MIF protein can be explained as an artificial modification which occurs through the chemical reduction processes of disulfite groups in the protein prior to MS/MS peptide identification (Boja and Fales, 2001; Chalkley et al., 2008).

The identified proteins in our biomarker panel serve as components of pro-inflammatory and inflammatory tumour microenvironment (Calandra and Roger, 2003; Chaly et al., 2000; Roth et al., 2003). The formation of a biomarker panel containing DEFA3, MIF and S100A8 proteins may enable assessment of differential expression patterns of proteins in whole tumour tissue involving both tumour cells and their microenvironmental interactions. The tumour microenvironment is composed of extracellular matrix (ECM) components, growth factors, cytokines, proteases and myriad of other molecules released from tumour cells, fibroblasts and infiltrating cells such as macrophages and lymphocytes (Witz and Levy-Nissenbaum, 2006). Gliomas

have been reported to harbour a heterogeneous cell population such as vascular cells, microglia, peripheral immune cells, and neural precursor cells (Charles et al., 2011). Recent studies have demonstrated the significance of molecular changes in tumour microenvironment associated with malignant phenotype (Seidel et al., 2010; Theys et al., 2009). The link between chronic inflammation and cancer have been extensively reviewed by several reports in gastric cancer (Polk and Peek, 2010), colorectal cancer (McLean et al., 2011), lymphoma (Smedby et al., 2006) and bladder cancer (de Martel and Franceschi, 2009).

Overexpression of DEFA3 protein was detected in non-responder patient group. Defensins are secreted by neutrophils as small, cationic and tridisulfide peptides demonstrating anti-microbial and immune-modulatory activities (Selsted and Ouellette, 2005). Defensins released by stimulated neutrophils induce the production of interleukin-8 (IL-8) which generates chemotactic signals via the activation of G protein-coupled Purinergic 2 Receptor Y6 (P2Y6) for the recruitment of additional neutrophils to the inflammation sites (Khine et al., 2006; van Wetering et al., 2002). Other researchers investigating signalling pathways downstream of P2Y6 activation have demonstrated that DEFA1-3 induced IL-8 release occurs in a Sarcoma Kinase (Src)-dependent manner through activated Extracellular Signal-Regulated Kinase-1 and -2 (ERK1/2) and Phosphatidylinositol 3 kinase/Protein Kinase B (PI3K/Akt pathways in monocytes (Grigat et al., 2007; Syeda et al., 2007). DEFA3 has been extensively investigated as a marker of inflammation and overexpression of DEFA in inflammatory diseases such as rheumatoid arthritis (Baillet et al., 2010), systemic lupus erythematosus (Bennett et al., 2003) and inflammatory bowel disease (Yamaguchi et al., 2009) have been reported. Inflammatory cytokines, IL-6, IL-8 and IL-1 β have been previously suggested to stimulate proliferation and survival of tumour cells (Singh and Varney,

1998; Tricot, 2002). Enhanced DEFA1-3 expression associated with increasing grade of malignancy has been reported in several tumour types including metastatic colorectal cancer (Albrethsen et al., 2006), breast cancer (Li et al., 2005b), cutaneous T-cell lymphoma (Escher et al., 2006), bladder cancer (Holterman et al., 2006) and invasive head and neck cancer (Roesch-Ely et al., 2007). While the role of DEFA1-3 in tumour growth remains controversial, anti-angiogenic activity in growth of human umbilical vein endothelial cells (HUVEC) and pathologic retinal neovascularization and also inhibitory effects on human papillomavirus infection have been reported by a number of studies (Chavakis et al., 2004; Economopoulou et al., 2005; Hubert et al., 2007). However, increased proliferation, invasiveness and motility of tumour cells due to potential influence of DEFA1-3 overexpression were evident in bladder cancer and renal cell carcinoma (Holterman et al., 2006; Muller et al., 2002).

MIF protein as one of the biomarkers identified in this study was found to be overexpressed in non-responder patients compared to responders. MIF is characterised as a pituitary-derived hormone as well as a cytokine acting in concert with Tumour Necrosis Factor-alpha (TNF- α) and Interferon-gamma (IFN- γ) in pro-inflammatory response (Bacher et al., 1996; Calandra et al., 1994). MIF secreted from corticotropic pituitary cells functions as counter-regulator of the anti-inflammatory and immunosuppressive effects of glucocorticoids (Calandra et al., 1995). Another unique function that differentiates MIF from other cytokines is its ability to catalyse the tautomerisation of 2-carboxy-2,3-dihydroindole-5,6-quinone (D-dopachrome) to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al., 1996). More recently, additive effects of MIF and its homolog D-dopachrome tautomerase (D-DT) have been shown to promote pro-angiogenic growth factors, VEGF and IL-8 expression in human lung adenocarcinoma cell lines (Coleman et al., 2008). Inhibition of MIF with ISO-1, an

inhibitor of the D-dopachrome tautomerase activity, resulted in an increase in the levels of MIF and its receptors Cluster of Differentiation 74 (CD74), Interleukin-8 receptor CXCR2 and Chemokine Receptor 4 (CXCR4) to promote tumour growth, however, loss of enzyme activity due to dose-dependent inhibition of cell proliferation reduced the growth rate of primary GBM cells *in vitro* (Baron et al., 2011). Another study using Hs 683 glioma cells (high endogenous levels of MIF) showed that MIF inhibitor ISO-1 sensitise cells to dexamethasone, a glucocorticoid used in the treatment of glioma in order to reduce tumour associated oedema, and exerts inhibitory effects on migration and invasion through activation of MAPK phosphatase 1 (MKP-1) pathway by dexamethasone (Lin et al., 2008; Piette et al., 2009). A significant association of high IL-8 expression with increased levels of MIF, as well as with higher histological grade, tumour recurrence, increased neovascularisation and poor prognosis have been recently corroborated in a study of 68 patients with high and low grade gliomas (Wang et al., 2011b). Moreover, Winner and co-workers discovered a new role for MIF related to hypoxia-dependent transcription in pro-tumorigenic processes (Winner et al., 2007). The authors suggested that MIF expression and secretion is increased by hypoxia induced factor-1 α (HIF-1 α) and MIF is required for the stabilisation of HIF-1 α to prevent its proteasome-mediated degradation in pancreatic adenocarcinoma cells (Winner et al., 2007). In addition, MIF has been shown to induce angiogenesis through activation of two major signalling pathways, PI3K-Akt and MAPK ERK Kinase/ Extracellular Signalling-regulated Kinase (MEK/ERK), using *in vivo* and *in vitro* models (Amin et al., 2003). In addition to its pro-tumorigenic activities including enhanced proliferation, migration and angiogenesis, immunosuppressive functions of MIF oppositely regulates anti-tumoral effects of NK and CD8⁺ cytotoxic T-cells which in turn mediates immune escape of gliomas (Mittelbronn et al., 2011).

S100A8 is the final member of our biomarker panel to be detected to be highly expressed in non-responder patients compared to responders using SELDI-TOF MS. S100 protein family was first identified in the brain and termed 'S100' because of their solubility in saturated ammonium sulphate solution (Moore, 1965). S100 proteins contain E- and F helices (EF hand) which selectively bind Ca^{2+} (Schafer and Heizmann, 1996). Monomers, heterodimers and also tetramers are formed by various types of S100 proteins in a calcium dependent manner (Vogl et al., 1999). In the present study, we could identify only the monomeric form of S100A8 protein. Strupat and co-workers reported heterodimeric (S100A8/A9) and even heterotetrameric (S100A8/A9)₂ forms of S100 proteins (Strupat et al., 2000). S100A8 monomer functions in recruitment of leukocytes into the inflammation sites upon pro-inflammatory stimuli and thus displays potent chemotactic activity for human myeloid cells (Hu et al., 1996). Inflammation associated overexpression of S100A8 has been suggested as a marker of the pathogenesis in inflammatory diseases such as juvenile idiopathic arthritis (Frosch and Roth, 2008), inflammatory bowel disease (Foell and Roth, 2004) and rheumatoid arthritis (Baillet et al., 2010). S100A8 and S100A9 are constitutively expressed in circulating neutrophils, myeloid-derived dendritic cells, platelets, osteoclasts, hypertrophic chondrocytes and monocytes while macrophages are positive for S100A8/A9 only in inflamed tissues (Goyette and Geczy, 2009). S100A8 and S100A9 proteins comprise 45% of the cytosolic proteins in neutrophils (Edgeworth et al., 1991). It has been reported that S100A8 and S100A9 induce expression of serum amyloid A (SAA), a chemoattractant that stimulates premetastatic pathways in lung cancer through activation of TLR4 and subsequent stimulation of NF- κ B signalling (Hiratsuka et al., 2008). In this pathway, these researchers also postulated that blocking SAA-TLR4 function in Lewis Lung Cancer (LLC) cells inhibits metastasis in pre-metastatic lungs

(Hiratsuka et al., 2008). In addition to TLR, S100A8 and S100A9 have been shown to interact with Receptor for Advanced Glycation End-products (RAGE) which induce NF- κ B signalling inferring a direct role for RAGE in chronic inflammation and cancer (Gebhardt et al., 2008). Ichikawa and co-workers further elucidate this mechanism in mouse model of colon cancer and reported RAGE-dependent activation of p38, ERK and the c-Jun N-Terminal Kinase (JNK) families which are components of MAPK pathway resulting in nuclear translocation of NF- κ B (Ichikawa et al., 2011).

As previously mentioned, overexpression of these biomarkers were strongly associated with poor prognosis of patient survival (Shimizu et al., 1999; Wang et al., 2011). In addition, S100A8 and MIF have been shown to play specific roles in mechanisms causing resistance to chemotherapeutics. Increased expression of S100A8 has been associated with drug resistance in breast cancer cells (Sommer et al., 2003). MIF-overexpressing prostate cancer cells demonstrated increased resistance to treatment with paclitaxel (Lin et al., 2005). In addition, in drug-resistant human metastatic colon carcinoma cell line HT-29, the presence of autocrine MIF-CXCR4 secretion loop has been reported to enhance the invasiveness of cancer cells (Dessein et al.).

The above mechanisms indicate overlapping functions of these proteins identified in our biomarker panel in pro-inflammatory and pro-tumorigenic processes. Correlation coefficients calculated to evaluate the relations between DEFA3, MIF and S100A8 showed positive correlation between expression patterns of these proteins. This association between expression profiles of the biomarkers may be due to crosstalk between these proteins and cytokines and also other components of the inflammatory pathway functioning through negative and positive feedback loops which in turn

possibly alter expression patterns of the proteins (Leng and Bucala, 2005; Riepl et al., 2010; Roth et al., 2003).

Following protein identification, expression levels of biomarkers were examined by immunoblotting in a subset of patient cohort. The results were in agreement with SELDI expression patterns indicating high level of DEFA3, MIF and S100A8 protein expression in non-responders compared to responders. The antibody selected to detect DEFA3 protein was specific to a conserved peptide sequence of precursor and mature DEFA3 forms. Unlike MIF, post-translational modification of DEFA protein is processed in a complex manner. Identified 94 aa long precursor form of DEFA3 (10.2 kDa) is known as preprodefensin and undergoes extensive cleavage processes which subsequently forms 29-30 aa long mature DEFA3. 75 aa (7.1kDa) and 56 aa (4.9 kDa) long major intermediate prodefensins are generated by the cleavage of sequence fragments from the N-terminal of the preprotein (Valore and Ganz, 1992). In the present study, overexpression of both preproprotein (10.2 kDa) and proprotein DEFA3 (7.1 kDa) in non-responder patient samples was confirmed by immunoblotting.

Early studies showed that alpha defensins are localised in cytoplasmic azurophil granules of neutrophils and three major alpha defensins, DEFA1-3, constitute 10-50% of the total cellular protein in mature neutrophils (Ganz et al., 1985; Rice et al., 1987). A study of five patients with oral squamous cell carcinoma indicated specifically tumour infiltrating neutrophils as the origin of DEFA1-3 expression (Lundy et al., 2004). However our immunoblotting results performed using GNS and glioma cell line lysates confirmed the presence of a defensin isoform with an approximate molecular weight of 14 kDa which thought to be the same peptide detected by Valore and co-workers in culture supernatants of human promyelocytic cell line (Valore and Ganz,

1992). In addition, amphiphilic dimer structures of mature defensins in the site of action can also explain the ~14 kDa isoform as a possible dimer formation by prodefensin (Hill et al., 1991). However, no detectable DEFA3 expression was observed in conditioned medium from GNS and glioma cell lines which could be due to absence of extracellular secretion of DEFA3 or low protein signals that might be beyond the limits of sensitivity of immunoblotting technique.

Preliminary results of immunohistochemical validation of DEFA3 expression in a new patient cohort are presented. DEFA3 expression was localised to the tumour-infiltrating neutrophils. There is some evidence in previous research showing high level of neutrophil infiltration into glioblastoma (Fossati et al., 1999).

High expression of MIF in non-responder patients was also verified by immunoblotting. MIF overexpression associated with poor prognosis and tumour aggressiveness has also been confirmed in numerous of other studies including melanoma (Shimizu et al., 1999), prostate cancer (Meyer-Siegler et al., 2002), breast cancer (Bando et al., 2002), hepatocellular carcinoma (Hira et al., 2005) and colon cancer (Legendre et al., 2003). Our subsequent immunoblotting analysis to detect MIF expression in GNS cells and glioma cell lines demonstrated expression of MIF in the absence of complete tumour microenvironment, confirming that tumour cells express MIF protein *in vitro*. We observed intense cytoplasmic immunostaining of MIF1 in our cohort. Strong staining of MIF in the palisading cells surrounding the necrotic foci was also observed. Our results confirm the findings from previous reports confirming tumour cell-intrinsic MIF localisation using immunohistochemistry (Mittelbronn et al., 2011; Munaut et al., 2002). Intense MIF staining in palisading cells around necrosis could be associated with HIF-1 α dependent induction of MIF (Baugh et al., 2006; Brat et al., 2004; Larsen

et al., 2008; Winner et al., 2007). In a previous study, immunohistochemical staining of glioma cell line LN18 and glioma tumour samples for MIF expression demonstrated a strong MIF localisation in the regions of vascular proliferation and/or the surrounding structures of necrosis which hypoxia-induced factors are predominantly expressed (Bacher et al., 2003). In the literature, MIF has also been reported to be expressed by several cells of the immune system including T cells, monocytes, macrophages, neutrophils, eosinophils, mast cells, basophils, monocytes, epithelial cells and endothelial cells (Calandra and Roger, 2003; Conroy et al., 2010).

High expression levels of S100A8 in non-responder patients were also confirmed by immunoblotting analysis. In a two dimensional difference gel electrophoresis (2D-DIGE) study of serum samples from 77 colorectal cancer and 22 control patients, elevated levels of S100A8 and S100A9 were suggested to be serological marker candidates that facilitate diagnosis of colorectal cancer (Kim et al., 2009). A microarray study demonstrated predictive value of four-gene signature; IL-1 β , S100A8, S100A9 and EGFR which were found to be correlated with risk of muscle invasive bladder cancer progression (Kim et al., 2011b). S100A8 overexpression associated with aggressive disease behaviour has also been reported in other cancer types such as, ovarian cancer (Cortesi et al., 2011; Ott et al., 2003) and cervical carcinogenesis (Koskimaa et al., 2010).

In a couple of studies, S100A8 or/and S100A9 expression have been shown to expressed at variable levels in cultured human adenocarcinoma and breast cancer cell lines (Arai et al., 2001; Bode et al., 2008). In contrast, our immunoblotting analysis showed no expression of S100A8 in GNS cells and glioma cell lines. In our cohort, S100A8 was localised to the macrophages. In the present study, no expression of

S100A8 was detected in tumour cells. Nevertheless, these findings are in agreement with previous studies demonstrating strong staining of S100A8 protein in infiltrating cells, macrophage and microglial cells in glioma (Deininger et al., 2001) and infiltrating leukocytes in epithelial skin carcinogenesis (Gebhardt et al., 2002), but not in tumour cells. Therefore in this study, it can be suggested that detected S100A8 protein expression in tumour samples might originate from tumour infiltrating macrophages and lymphocytes which were present in the tumour microenvironment. In the study by Vogl *et al.* (2007), S100A8 and S100A9 released by phagocytes of activated-monocytes have been demonstrated to interact with Toll Like Receptor-4 (TLR4) / Myeloid Differentiation Factor-2 (MD2) complex and activate Interleukin-1 receptor-associated kinase-1 (IRAK-1) and Nuclear Factor- κ B (NF- κ B) which in turn increase TNF- α expression, hence augment inflammation-associated conditions. In a study which investigated the infiltration of monocytic cells into glioma spheroids demonstrated that peripheral blood monocytes infiltrate A172 and U118 glioma cell spheroids *in vitro* and also that labelled peritoneal rat macrophages infiltrate into the intracerebral glioma following intra-arterial and intravenous injection (Strik et al., 2006). The same researchers also reported strong expression in infiltrating cells stained staining for late inflammatory macrophage, S100A8 and S100A9 proteins (Strik et al., 2006).

We demonstrated overexpression of a panel of three biomarkers in non-responder patient group which could potentially serve in predicting response outcomes of the patients to the standard therapy of glioma. Although prior biomarker discovery studies examined tumour tissue, CSF, and serum of glioma patients over the past 10 years, our findings describe the first panel of multiple biomarkers identified using SELDI-TOF MS in human tumour specimens of an extensive glioma cohort. Our finding also contributes to our understanding of the effect of inflammatory proteins on the

interactions between tumour cells and their microenvironment. Identification of these predictive biomarkers which play roles in inflammation-associated malignancy might provide a novel approach and implications in foreseeing response of patients to the standard therapy.



CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

Glioblastomas represent formidable challenges in terms of therapy, quality of life and survival. Prognosis of patients diagnosed with glioblastoma confers a median survival time of just 15 months, even after aggressive therapy with multiple modalities including total tumour resection by surgery, radiotherapy and chemotherapy, specifically temozolomide (Stupp et al., 2005). The majority of patients are unresponsive to chemotherapy regimens and resistance to treatment portends a dismal prognosis. The unfortunate fact is, that although a small portion of the patients respond to treatment eventually experience tumour recurrence. The underlying mechanisms involved in the differential drug response of glioblastoma are not sufficiently elucidated. In this thesis, we investigated the association between chemotherapy resistance and the expression levels of single biomarkers known to be potential targets of chemotherapeutic agents. In addition to our single biomarker approach, a global proteomic profiling method was utilised to discover potential predictive or prognostic biomarkers to associated with treatment response.

Etoposide is a common drug used in combination with carboplatin to treat recurrent glioblastoma. I examined the levels of TopoII α expression and its involvement in

relative resistance to etoposide (Chapter 3). Previous studies in breast cancer have investigated the role of TopoII α in drug response comprehensively and have shown a tight association with etoposide response (Oakman et al., 2009). In the present study, we assessed the endogenous expression levels of TopoII α in p53-mt and p53-wt glioma cell lines. TopoII α expression was also analysed after exposure to etoposide treatment to monitor etoposide-dependent alteration in the levels of TopoII α . *In vitro* models used in this study showed the regulatory effect of p53 mutations in TopoII α expression and confirmed an association between mutant p53 and over-expression of TopoII α expression. Despite this, no clear association was established between endogenous expression levels of TopoII α in glioma cell lines and their response to etoposide (IC₅₀). This finding is consistent with reports in the literature which have also found that TopoII α as a marker alone is not sufficiently predictive of clinical outcome of leukemia and small-cell lung cancer (Ceppi et al., 2008; Kaufmann et al., 1994; Rossman et al., 2011; Uggla et al., 2007). Increasing evidence supports the presence of mutations in the TopoII α gene can result in drug resistance. A phase II trial conducted in glioblastoma patients examined genomic alterations in TopoII α gene and revealed alterations in the copy number of TopoII α in only a small number of patients (Franceschi et al., 2004).

Ultimately, treatment response is determined by a myriad of other factors. I demonstrated the response of glioma cell lines to etoposide appeared to be a complex mechanism that might comprise molecular changes in other effectors of response pathways possibly including BH3-only protein BID, MRPs, Gli2 DNA-PK dependent repair mechanisms (Chen et al., 2009a; Cui et al., 2010; Jin et al., 1998; Maas et al., 2011).

A second single biomarker examined in this thesis was NG2 expression levels which were measured in glioma cell lines to evaluate its contribution to drug resistance (Chapter 4). High expression levels of NG2 are associated with chemoresistance in glioma cells and play a role in potentiating cell survival, proliferation and migration (Chekenya et al., 2008). There is some evidence that NG2 co-localises with $\beta 1$ integrins (Makagiansar et al., 2007; Makagiansar et al., 2004), therefore in this study we examined the response of the glioma cell lines to the integrin ($\alpha v\beta 3$ and $\alpha v\beta 5$) targeting peptide-like molecule, cilengitide. Variable expression levels of NG2 were found in the glioma cell lines. These results also demonstrated differential sensitivity to cilengitide treatment. Interestingly, low-NG2 expressing cell lines (T98G, M059K and M059J) showed higher resistance while the cell lines with high endogenous NG2 expression (A172 and U-87 MG) were found to be more sensitive to cilengitide. These results are in contrast to a study which indicated that high NG2 expression levels were correlated with increased resistance to etoposide, doxorubicin and vincristine (Chekenya et al., 2008). Interestingly, our results in Chapter 3 showed that cilengitide-resistant cell lines were also detected to be resistant to treatment with etoposide. This discrepancy can be possibly explained by the complex functional interactions between NG2 and various types of integrins, predominantly $\beta 1$ integrins. Further experiments conducted to suppress NG2 expression in A172 cells showed no considerable change in cilengitide response suggesting the involvement of other factors in mechanisms associated with chemotherapy response. Future studies are also needed to elucidate the function of NG2 in chemotherapy response by examining expression levels of a panel of integrin complexes including $\alpha 3\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 4\beta 1$ which were previously reported to be associated with cilengitide response or signalling partners to NG2 (Eisenmann et al., 1999; Makagiansar et al., 2007; Makagiansar et al., 2004; Smith, 2003; Yang et al.,

2004a). Understanding this multifaceted relationship may help to develop strategies to overcome chemotherapy resistance in glioblastoma.

The final single biomarker I looked at was the contribution of MGMT promoter methylation and protein expression to chemoresistance in glioblastoma (Chapter 5). Methylation status of MGMT promoter was detected using both MSP and a quantitative analysis method, pyrosequencing. The methylation status of MGMT promoter is considered of paramount importance in predicting prognosis and to concurrent radiotherapy and temozolomide therapy and response in glioblastoma patients, due to its ability of reversing DNA damage caused by TMZ (Kaina et al., 2007). As a potential biomarker, MGMT has been widely studied and suggested to be an independent prognostic factor of favourable outcome (Hegi et al., 2005; Stupp et al., 2005). The results presented in this study showed that methylation of the MGMT promoter was detected in all glioma cell lines as confirmed by both MSP and pyrosequencing. This is not reflective of the incidence of MGMT methylation in the patient population (~35%).

It is generally agreed that commercial cell models provide valuable information in understanding complex nature of drug-resistance in glioblastoma by allowing investigation of overexpression and knockdown of certain elements in these pure neoplastic cell populations (Chekenya et al., 2008; Kim et al., 2011a; Matsumoto et al., 2005; Mohri et al., 2000; van Niftrik et al., 2010). However, the use of commercial cell lines has some limitations which need to be considered. Although our commercial cell lines were purchased from the ATCC, high passage numbers are common which result in changes to their proliferative capacity and epigenetic pattern. For example, all of our cell lines were MGMT promoter methylated which does not reflect what is observed clinically (around 30% of patients are MGMT methylated). The commercial cell lines

grow in monolayers on plastic surfaces, thus neglecting the contribution of tumour microenvironment. A 3-dimensional co-culture systems integrating the impact of tumour microenvironment would be a suitable culture models to investigate chemoresistance in glioblastoma. Therefore it would also be worthwhile to examine the MGMT promoter methylation and TMZ-response of glioma neural stem cells (GNS)-derived from primary glioblastomas which have only been recently become available after the completion of this study.

Previous studies have reported that the incidence of MGMT promoter methylation was between 30 and 55% in the patient population (Brell et al., 2005; Esteller et al., 2000; Hegi et al., 2005; Nakamura et al., 2001; Paz et al., 2004). We tested patient samples recruited from Royal North Shore Hospital (RNSH) for MGMT promoter methylation and found an incidence of 26% consistent with other studies (Nakamura et al., 2001; Paz et al., 2004). Although the correlation of MGMT promoter methylation with survival and response outcomes were found to be non-significant, a small survival advantage was observed in the patient group with methylated MGMT promoter. Our cohort was too small to be representative of the previous reports investigating the prognostic power of MGMT promoter methylation which have implicated a survival benefit for the patients with methylated MGMT promoter (Hegi et al., 2005; Stupp et al., 2009; van den Bent et al., 2009a; Wick et al., 2009). MGMT protein expression also demonstrated no significant association with patient response to standard therapy and survival outcome. In addition, protein expression levels were not found to be correlated with MGMT promoter methylation. Several research groups also indicated the lack of association between MGMT protein expression levels and promoter methylation status using immunohistochemistry (Brell et al., 2005; Jeuken et al., 2007; Preusser et al., 2008; Rodriguez et al., 2008). In conclusion, the work in this study showed that MGMT

promoter methylation could be determined using both MSP and pyrosequencing. No correlation of MGMT promoter methylation with response to therapy was established, whereas, a small survival benefit was determined in patients with methylated MGMT promoter. It is also concluded that MGMT promoter methylation status alone is not completely sufficient as a predictive factor. From the point of view of future studies, the work represented in this study require further investigation in a larger cohort to better understand the discordance between MGMT promoter methylation and response to TMZ treatment; and MGMT promoter methylation and protein expression.

Due to multiple complicating factors interfering with chemotherapy resistance in glioblastoma, new strategies for the discovery of effective biomarkers are demanded. As discussed earlier, MGMT is the only potential marker that has been suggested to be a predictive of patient response and survival outcome. However, inconsistencies exist in the literature regarding its ability of correctly predicting patient response (Hegi et al., 2005; Stupp et al., 2009; Wick et al., 2009). In addition, no significant correlation was detected between MGMT promoter methylation and patient response in our relatively small patient cohort. Therefore, we developed a proteomics approach to examine protein profiles from 42 tumour specimens from glioblastoma patients (Chapter 6).

The patients were dichotomised as responders and non-responders based on PFS6, and LTS and STS (grouped according to above or below the median overall survival [13.8 months]). SELDI-TOF-MS technology was used to identify novel small molecular weight proteins associated with poor response to standard therapy. Data consolidated from four different chemically treated surfaces indicated 44 and 39 peaks were found to be significantly differentially expressed between NR vs. R, and STS vs. LTS groups, respectively. BLR analysis of those selected peaks generated powerful

biomarker panels. A combination of three peaks (10.2, 12.3 and 10.8 kDa) revealed a strong prediction model with 100% sensitivity and specificity in response group. Discriminative ability of two protein peaks (12.3 and 3.6 kDa) identified for survival group using BLR analysis showed a powerful AUC value, 0.917 (sensitivity and specificity, 84.2%). Combined analysis of protein peaks demonstrated a stronger AUC when compared with their individual AUCs. The final panel including three proteins with known molecular weights, 10.2, 12.3 and 10.8 were identified to be DEFA3, MIF and S100A8. All three proteins were up-regulated in non-responders. An interesting finding from this study was the significant up-regulation of MIF protein both in the non-responder and STS cohorts. All of the identified proteins are known to serve as key elements of pro-inflammatory and inflammatory tumour microenvironment (Calandra and Roger, 2003; Chaly et al., 2000; Roth et al., 2003). The association between chronic inflammation and cancer have been extensively reviewed by a number of reports in numerous cancers such as gastric cancer (Polk and Peek, 2010), colorectal cancer (McLean et al., 2011), lymphoma (Smedby et al., 2006) and bladder cancer (de Martel and Franceschi, 2009). Our findings are in agreement with earlier studies suggesting overexpression of DEFA3 (Albrethsen et al., 2006; Muller et al., 2002; Roesch-Ely et al., 2007), MIF (Mittelbronn et al., 2011; Wang et al., 2011b) and S100A8 (Baillet et al., 2010; Foell and Roth, 2004) in various cancers and inflammatory diseases. The present results were verified by detecting protein expression using immunoblotting in training patient cohort. We also examined expression of these biomarkers *in vitro* glioma cell models (commercial glioma cell lines and GNS cells). MIF and a different isoform of DEFA3 were found to be expressed in tumour cells while no expression of S100A8 detected *in vitro*. In addition, conditioned medium of glioma cell lines and GNS cells were analysed for the expression of the biomarkers.

However, no detectable level of signal from any of the proteins was obtained using immunoblotting. It would be valuable to investigate the level of extracellular secretion of DEFA3 and MIF factors by utilising more specific techniques such as ProteinChip antibody capture method or ELISA which is based on indirect chemical or radioactive detection of the peptides.

In a study using Hs 683 glioma cells (high endogenous MIF expressor) indicated that MIF inhibitor ISO-1 sensitised cells to dexamethasone which is a glucocorticoid used in the treatment of glioma to reduce tumour associated oedema and exerts inhibitory effects on migration and invasion via dexamethasone-activated MAPK phosphatase 1 (MKP-1) pathway (Lin et al., 2008; Piette et al., 2009). In a recent study, myeloid-derived suppressor cells secreting high levels of S100A8/9 were shown to co-localise with tumour cells in metastatic sites and S100A9 null mice showed remarkably reduced tumour growth, metastasis and resulted in low levels of infiltrative CD11b⁺Gr1⁺ myeloid progenitor cells within the tumours and sites of metastasis in colon cancer (Ichikawa et al., 2011). Interaction of S100A8 as a ligand of receptor for advanced glycation end-products (RAGE) indicated the role these molecules in chronic inflammation and tumour promotion of skin carcinogenesis and in inflammation-associated joint and vascular damage in rheumatoid arthritis (Chen et al., 2009b; Gebhardt et al., 2008). It will be important for future studies to integrate the findings presented in this thesis with *in vivo* models to explore the functions of these biomarkers.

Confirmation of DEFA3, MIF and S100A8 protein expression was conducted in a validation cohort including 66 tumours from newly diagnosed glioblastoma patients using IHC. IHC staining of tumour sections for DEFA3, S100A8 and MIF-specific antibodies confirmed that DEFA3 and S100A8 were expressed by neutrophils and

macrophages. MIF1 was expressed by tumour cells and was localised to the cytoplasm. The evaluation and scoring of the samples are currently under evaluation. Tissue specimens were difficult to obtain from pathology and I still have not secured the original FFPE specimens of the cohort we used in the SELDI-TOF experiment. Analysis of individual and combined contributions of these biomarkers in predicting response and survival outcome of the glioblastoma patients in validation cohort will further confirm the validity of these biomarkers. Overall in this study, we demonstrated a unique model to differentiate responders from non-responders and LTS from STS with high predictability. It is clear from this work that patients expressing high levels of identified pro-inflammatory markers, DEFA3, MIF and S100A8 are poor responders. Future studies are also needed for the identification of protein peaks in additional biomarker panels which were found to be overexpressed in responders and LTS.

The findings presented in this thesis may expand the present glioblastoma literature. Ultimately, information in this thesis has strengthened the knowledge which might be utilised for future assessment and management of individuals with glioma by suggesting a novel algorithm to differentiate between responder and non-responder, and LTS and STS with glioblastoma.

APPENDIX A

Materials

Chemicals	Suppliers
Hepes, free acid, 1 kg	#AM0511 Amresco, Solon, OH, USA
10% neutral buffered formalin 5 L	#JJ018 Fronine (Thermo Fisher), NSW, Australia
18S rRNA probe dye: VIC-MGB (linked to JOE)	#4319413E Applied Biosystems, Mulgrave, VIC, Australia
Acetonitrile gradient 240 nm/far UV HPLC gradients	#AC03294000 Scharlau Chemie, Barcelona, Spain
AcroPrep 96 350 µl filter plate with Mustang S ion exchange media, natural housing	#5048 Pall Corporation, Port Washington, NY, USA
Acrylamide:bisacrylamide 19:1 ultra pure grade 40%	#0496 Amresco, Solon, OH, USA
Allstars™ negative control siRNA (20 nmol)	#1027281 Qiagen, Hilden, Germany
Amersham™ ECL plus western blotting detection system	#RPN2132 GE Healthcare, Buckinghamshire, UK
Amersham™ Hybond™ ECL nitrocellulose membrane	#RPN203D GE Healthcare, Buckinghamshire, UK
Ammonium persulfate ≥98%	#A3678 Sigma-Aldrich, St Louis, MO, USA
Antibody, mouse IgG2A (Negative Control), 1 ML	#X094301 Dako, Glostrup, Denmark
Antibody, goat polyclonal anti-mouse, HRP-linked	#P0447-01 Dako, Glostrup, Denmark
Antibody, goat polyclonal anti-rabbit, HRP-linked	#7074 Cell Signalling Technology
Antibody, rabbit Immunoglobulin fraction (Negative Control), 2 ML	#X0903 Dako, Glostrup, Denmark
Antibody, rabbit polyclonal anti-goat, HRP-linked	#P0449-01 Dako, Glostrup, Denmark
Antibody, rabbit polyclonal anti-sheep , HRP-linked	#P0163-02 Dako, Glostrup, Denmark
Anti-human, MGMT clone MT3.1 mouse monoclonal antibody	#MAB16200 Millipore Corporation, Billerica, MA, USA
Anti-human, DEF-3 mouse monoclonal antibody (IHC)	#T-1034 BMA Biomedicals, Augst, Switzerland
Anti-human, alpha Defensin (1+2+3) goat polyclonal Antibody (WB)	#ab99504 Abcam, Cambridge, UK
Anti-human, α-tubulin mouse monoclonal antibody clone DM1A 0.2mL	#T9026 Sigma-Aldrich, St Louis, MO, USA
Anti-human, Topoisomerase IIα mouse monoclonal antibody, clone Ki-S1	#M7186 Dako, Glostrup, Denmark

Anti-human, MIF goat polyclonal antibody (WB)	#AB-289-PB R&D Systems, Minneapolis, MN, USA
Anti-human, MIF mouse monoclonal antibody (IHC)	#MAB289 R&D Systems, Minneapolis, MN, USA
Anti-human, MRP8 rabbit polyclonal antibody (IHC)	#ab92331 Abcam, Cambridge, UK
Anti-human, NG2 Chondroitin Sulfate Proteoglycan rabbit polyclonal antibody	#AB5320 Millipore Corporation, Billerica, MA, USA
Anti-human, S100A8 sheep polyclonal antibody (WB)	#AF 4570 R&D Systems, Minneapolis, MN, USA
Pierce® BCA protein assay kit-RAC	#PIE23250 Thermo Scientific, Waltham, MA, USA
Bicine, high purity grade	#Q149-1KG Amresco, Solon, OH, USA
Biotin blocking system	#X0590 Dako, Glostrup, Denmark
Bis-Tris, ultra pure grade	#0715-500G Amresco, Solon, OH, USA
Bond polymer refine detection kit	#DS9800 Leica Microsystems, VIC, Australia
Boric acid, 1 kg	#B7901 Sigma-Aldrich, St Louis, MO, USA
Bovine serum albumin (BSA)	#9998 Genesearch 50g
Bromophenol blue	#IB74040 International Biotechnologies Inc., New Haven, CT, USA
Buffer 10X PCR II & MgCl ₂	#N8080010 Applied Biosystems, Foster City, CA, USA
Cell line nucleofector ® kit T	#VCA-1002 Lonza Australia, Pty. Ltd. Waverley, VIC, Australia
CellTiter 96® AQueous MTS reagent, powder	#G1112 Promega Corporation, Madison, WI, USA
CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1 propanesulfonate	#C5070 Sigma-Aldrich, St Louis, MO, USA
Chloroform GR analytical Grade 2.5L	#1024452500 Crown Scientific, Minto, NSW, Australia
Cilengitide	Merck KGaA, Darmstadt, Germany
Complete protease inhibitor tablets	#11697498001 Roche, Indianapolis, IN, USA
Copper(II) sulfate	#C1297 Sigma-Aldrich, St Louis, MO, USA
Culture medium, PBS powder, Ca-Mg-free	#21600-010 Invitrogen, Carlsbad, CA, USA
Dimethyl sulfoxide (DMSO)	#154938 Sigma-Aldrich, St Louis, MO, USA
DNA ladder, pUC19/HpaII	#SM0223 MBI Fermentas, Vilnius, Lithuania
DNA polymerase high fidelity, platinum® Taq	#11304-029 Invitrogen, Carlsbad, CA, USA

DNA polymerase, AmpliTaq Gold®12x250 units with bufferII and MgCl ₂ solution	#N8080245 Applied Biosystems, Foster City, CA, USA
DNA polymerase, Platinum Taq	#10966-018 Invitrogen, Carlsbad, CA, USA
DNase buffer 10x recover all total nucleic acid isolation kit 240 µl	#F8166G AM1975 Applied Biosystems/Ambion, Austin, TX, USA
DNase recover all total nucleic acid isolation kit 160 µl	#2240G AM1975 Applied Biosystems/Ambion, Austin, TX, USA
dNTP set 100 mM	#10297-018 Invitrogen, Carlsbad, CA, USA
DTT DL-Dithiothreitol pro-pure proteomics grade	#M109-5G Amresco, Solon, OH, USA
Dulbecco's Modified Eagle Medium (DMEM) powder, high glucose	#12800017 Invitrogen, Carlsbad, CA, USA
Dulbecco's Modified Eagle Medium (DMEM) powder, low glucose	#31600-034 Invitrogen, Carlsbad, CA, USA
Dulbecco'S Phosphate-Buffered Saline (D-PBS) powder	SKU #21300-025 Invitrogen, Carlsbad, CA, USA
EDTA Disodium salt, dihydrate (biotechnology grade) 500 g	#AM0105 Amresco, Solon, OH, USA
Envision™+ Dual link detection kit, 10X	#K406189 Dako, Glostrup, Denmark
ENZYME, RNaseZAP™, 250 ml	#R2020 Sigma-Aldrich, St Louis, MO, USA
Eosin stain 2 litres	#II014 Fronine (Thermo Fisher), NSW, Australia
Eppendorf safe-lock microcentrifuge tubes volume 2.0 ml, Amber	#T2920-500EA Sigma-Aldrich, St Louis, MO, USA
Ethanol, absolute 200 proof, 500 ml	#E7023 Sigma-Aldrich, St Louis, MO, USA
Etopophos® (Etopophos phosphate)	Bristol-Myers Squibb Co., New York, NY, USA
EZ DNA methylation gold kit	#D5005 Zymo Research, Irvine, CA, USA
Fetal bovine serum (FBS), 500 ml, LOT No. 8J0001	#12003C Sigma-Aldrich, St Louis, MO, USA
Glycerol, AJAX	#24225LGL Crown Scientific, Minto, NSW, Australia
Haematoxylin stain, Harris solution 500 ml	#II001 Fronine (Thermo Fisher), NSW, Australia
HiMark* pre-stained high molecular weight protein standard	#LC5699 Invitrogen, Carlsbad, CA, USA
Human brain reference RNA(1 mg/ml)(200 µg)	#6051 Applied Biosystems/Ambion, Austin, TX, USA
Human HCT 116 DKO methylated DNA standard	#D5014-2 Zymo Research Corp., CA, USA
Human orbital frontal cortex brain total (1 mg/ml)(50 µg) RNA	#AM6786 Applied Biosystems/Ambion, Austin, TX, USA
Human parietal cortex posterior brain total (1 mg/ml)(50 µg)	#AM6788 Applied Biosystems/Ambion, Austin, TX, USA

Human parietal cortex superior brain total RNA (1 mg/ml)(10 µg)	#6790 Applied Biosystems/Ambion, Austin, TX, USA
In situ cell detection kit, POD	#11684817910 Roche Diagnostics, QC, Canada
Isopropanol, propan-2-ol-UNILAB	#425-2.5L GL Bacto Laboratories, Mt Pritchard, NSW, Australia
Lithium chloride 7.5M	#AM9480 Applied Biosystems/Ambion, Austin, TX, USA
LSAB™+ Kits, universal	#K0690 Dako, Glostrup, Denmark
Lyophilisation vacuum, cryodos freeze drier	# 22960 Telstar Industrial, Terrassa, Spain
Magnesium chloride, anhydrous, ≥98%	#M8266 Sigma-Aldrich, St Louis, MO, USA
Methanol, CHROMASOLV	#34860 Sigma-Aldrich, St Louis, MO, USA
Methanol, UNIVAR plastic bottle, analytical reagent	#318-2.5L PL AJAX(Lomb Scientific-Thermo Fisher), NSW, Australia
COATASIL glass treatment solution	#2293-500ML AJAX(Lomb Scientific-Thermo Fisher), NSW, Australia
Molecular grade agarose	#161-3102 Bio-Rad Laboratories, Hercules, CA, USA
Mini-Protean TGX 4-20% gel (10-well)	#456-1093 Bio-Rad Laboratories, Hercules, CA, USA
Mini-ProteanTGX 4-15% gel (15 well)	#456-1083 Bio-Rad Laboratories, Hercules, CA, USA
MycoAlert Kit	#LT07-318 Cambrex Biosciences, Rocklands, ME, USA
N N N' N'-Tetramethylethylenediamine (TEMED) ULTRA PURE GRADE, 25ML	#AM0761 Amresco, Solon, OH, USA
Nucleofector™ II Device	#AAD-1001 Lonza, Walkersville, MD, USA
NuPAGE LDS sample buffer	#NP0007 Invitrogen, Carlsbad, CA, USA
NuPAGE MES SDS running buffer (20X)	#NP0002 Invitrogen, Carlsbad, CA, USA
NuPAGE Novex 3-8% Tris-Acetate mini gels 1.0mm, 12-well	#EA03752BOX Invitrogen, Carlsbad, CA, USA
NUPAGE Novex 4-12% Bis-Tris Gel 1mm, 12-well	#NP0322BOX Invitrogen, Carlsbad, CA, USA
NuPAGE Novex Tris-Acetate SDS running buffer	#LA0041 Invitrogen, Carlsbad, CA, USA
NuPAGE sample reducing buffer (10X)	#NP0004 Invitrogen, Carlsbad, CA, USA
NuPAGE® MOPS SDS running buffer	#NP0001 Invitrogen, Carlsbad, CA, USA
NuPAGE® Tris-Acetate SDS running buffer	#LA0041 Invitrogen, Carlsbad, CA, USA
Paraformaldehyde	#P6148-500G Sigma-Aldrich, St Louis, MO, USA
PBS (20X) liquid concentrate 1L	#AME703 Amresco, Solon, OH, USA

Phenazine methosulfate (PMS)	#P-9625 Sigma-Aldrich, St Louis, MO, USA
Ponceau S solution	#P7170 Sigma-Aldrich, St Louis, MO, USA
Protein standard, BenchMark™ protein ladder (Unstained)	#10747-012 Invitrogen, Carlsbad, CA, USA
Protein standard, Precision Plus Kaleidoscope, 500µl	#161-0375 Bio-Rad Laboratories, Hercules, CA, USA
Protein standard, SeeBlue Pre-Stained Plus	#LC5925 Invitrogen, Carlsbad, CA, USA
Proteinase K 100 mg	#03115879001 Roche, Indianapolis, IN, USA
Proteinase K-ready to use 110 ml	#S302030 Dako, Glostrup, Denmark
ProteoMass™ insulin MALDI-MS individual standard	# I6279 Sigma-Aldrich, St Louis, MO, USA
PyroMark Q96 CpG MGMT	#972032 Qiagen, Hilden, Germany
QIAamp DNA mini kit	#51304 Qiagen, Hilden, Germany
QIAamp® DNA FFPE tissue kit (50)	#56404 Qiagen, Hilden, Germany
RC DC protein assay kit II	#500-0122 Bio-Rad Laboratories, Hercules, CA, USA
Silencer select pre-designed siRNA	Cat#4392420 Ids:s3651/s3652/s3653 Applied Biosystems/Ambion, Austin, TX, USA
Sinapic Acid 98% titration 3,5-Dimethoxy-4-hydroxycinnamic acid, 4-Hydroxy-3,5-dimethoxy-cinnamic acid	#D7927 Sigma-Aldrich, St Louis, MO, USA
Skim milk powder	Coles Supermarkets, Glen Iris, VIC, Australia
Sodium acetate (Anhydrous) 250 g	#S2889 Sigma-Aldrich, St Louis, MO, USA
Sodium citrate dihydrate 1KG-K	#W302600 Sigma-Aldrich, St Louis, MO, USA
Sodium dodecyl sulphate, SDS 250 G	#L4509 Sigma-Aldrich, St Louis, MO, USA
Substrate, liquid DAB+ chromogen kit, 110 ml	#K346811 Dako, Glostrup, Denmark
Superscript III first strand synthesis system, 10000 U	#18080-051 Invitrogen, Carlsbad, CA, USA
SYBR® safe DNA gel stain	#S33102 Invitrogen, Carlsbad, CA, USA
SYPRO ruby protein stain	#S12000 Invitrogen, Carlsbad, CA, USA
Taqman gene expression assays TOP2A probe (Linked to FAM)	Hs00172214_m1 Applied Biosystems, Foster City, CA, USA
Taqman gene expression assays CSPG4 probe (NG2) (Linked to FAM)	Hs00426981_m1 Applied Biosystems, Foster City, CA, USA
TaqMan® universal PCR master mix, No AmpErase® UNG	#4324018 Applied Biosystems, Foster City, CA, USA

TaqMan® gene expression master mix	#4369016 Applied Biosystems, Foster City, CA, USA
Target retrieval solution, citrate pH 6.0 500 ml	#S2369 Dako, Glostrup, Denmark
Target retrieval solution, pH 9.0 concentrate Tris/EDTA 500 ml	#S2367 Dako, Glostrup, Denmark
Temodar®	Schering-Plough Corporation, Kenilworth, NJ, USA
Tissue embedding cassette (white), Histosette	#M492-2 Simport Plastics, Beloeil, QC, Canada
TRI reagent	#T9424 Sigma-Aldrich, St Louis, MO, USA
Trifluoroacetic acid (TFA), CHROMASOLV for HPLC	#302031 Sigma-Aldrich, St Louis, MO, USA
Tris hydrochloride, ultra pure grade	#0234 Amresco, Solon, OH, USA
Tris, ultra pure grade	# 0497 Amresco, Solon, OH, USA
Triton X-100, 250 ml	#T8787 Sigma-Aldrich, St Louis, MO, USA
Trypsin/EDTA (0.25%), 500 ml	#T4049 Sigma-Aldrich, St Louis, MO, USA
Tween 20, 500 ml	#P1379 Sigma-Aldrich, St Louis, MO, USA
Ultramount #4 100 ml squeeze tube	#II065C Fronine (Thermo Fisher Scientific), NSW, Australia
Unmethylated DNA epitect human control DNA	#59665 Qiagen, Hilden, Germany
Urea, ultra pure, 2.5 kg	#0568 Amresco, Solon, OH, USA
Vacuum manifold, multi-well plate	#5017 Pall Corporation, Port Washington, NY, USA
Water for irrigation, 1000 ml	#2F7114 Baxter Healthcare, Deerfield, IL, USA
Xylene, AR 2.5 L	#XA003-2.5L Chem-Supply, Port Adelaide, SA, Australia
GeneRuler™ 1 kb DNA ladder	#SM0311 Thermo Scientific, Waltham, MA, USA

Equipments	Suppliers
250 x 4.6mm Jupiter® 5 µm, C18 300Å ⁰ LC column	#00G-4053-E0 Phenomenex, Torrance, CA, USA
Automated pipetting system, epMotion 5070	#5070 000.000 Eppendorf, Hamburg, Germany
Autostainer plus	Dako, Glostrup, Denmark
BD Plastipak™ syringe hypodermic 10 ml Luer slip	#302143 BD Medical, North Ryde, NSW, Australia
BD Plastipak™ syringe hypodermic 30 ml Luer slip	#301229 BD Medical, North Ryde, NSW, Australia
BD Plastipak™ syringe hypodermic 5 ml Luer slip	#302130 BD Medical, North Ryde, NSW, Australia
Biological safety cabinet class II	Email Air Handling, Sydney, NSW, Australia
Biopsy pads, black (500)	#TT030L Lomb Scientific (Thermo Fisher), NSW, Australia
Bond X automated immunostainer	Leica Microsystems, VIC, Australia
Cell Scraper, 24 cm length, 12mm width	#TPP99002 MIDSCI, St Louis, MO, USA
Eppendorf centrifuge 5424	Eppendorf, Hamburg, Germany
Eppendorf refrigerated centrifuge 5417R	Eppendorf, Hamburg, Germany
Chromatography system: HPLC	Waters pump model 510, Waters 486 Tunable Absorbance Detector, Waters Corporation, Milford, MA, USA
Clear autosampler shell vial 2ml (12x32mm)	#60831-1231 KIMBLE® Glass, Inc., Vineland, NJ, USA
Cover slip, haemocytometer	#SCH80001.21 Schott (Thermo Fisher Scientific), NSW, Australia
Coverglass, 50x22 mm coverslips	#HD LD22501.01P3 HD Scientific Supplies P/L, Wetherill Park BC, NSW, Australia
Culture flask, 75 cm ² , easy filter flask	#NUN156499 Thermo Scientific, Waltham, MA, USA
Culture plate, 6-well 90 X 150 mm, NUNC	#140675 Thermo Scientific, Waltham, MA, USA

Dako pen	#S2002 Dako, Glostrup, Denmark
Durapore membrane filters 0.45 µm HV	#HVLPO4700 Millipore Corporation, Billerica, MA, USA
Eppendorf MixMate	Eppendorf, Hamburg, Germany
Filter Paper 3MM	Whatmann International Ltd., Maidstone, UK
Filter, 32mm 0.2µM, Millex	#SLGP033RS Millipore Corporation, Billerica, MA, USA
Filter, Vacuicap 60	#4632 Pall Corporation, Port Washington, NY, USA
Fraction collector FRAC-100	Pharmacia Fine Chemicals (Amersham BioSciences/GE GE Healthcare), Buckinghamshire, UK
FUJIFILM image reader FLA-3000	FUJI Photo Film Ltd, Tokyo, Japan
Gel Doc™ XR	Serial #720BR/01637 Bio-Rad Laboratories, Hercules, CA, USA
Glass plates, Bio-Rad® MINI Protean® III, short plate	#GBU3S-5 GelCompany, San Francisco, CA, USA
Heat block, Termastat plus	Eppendorf, Hamburg, Germany
HPLC 2.5 ml syringe	#008500 2.5MDR-GT SGE Analytical Science, Melbourne, Australia
Kimwipes	#4103 Kimberly-Clark, Irving, TX, USA
Mini trans-blot electrophoretic transfer cell	#170-3930 Bio-Rad Laboratories, Hercules, CA, USA
Multi function 3D rotator	#PS-M3D Grant Instruments, Shepreth, Cambridgeshire, UK
Multi gauge software V3.0	FUJI Photo Film Ltd, Tokyo, Japan
Nunc microwell plates polystyrene flat bottom	#167008 Thermo Scientific, Waltham, MA, USA
Nunclon surface (Delta) well plates flat bottom polystyrene	#140675 Thermo Scientific, Waltham, MA, USA
Orbital shaker	Paton Scientific P/L, SA, Australia

PAWS statistics 18, SPSS statistics software	IBM® Company, Chicago, IL, USA
PowerPac basic™ power supply	#164-5050 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip CM10 arrays	#C5730075 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip data manager software 4, enterprise edition	#SW3-040030 Bio-Rad Laboratories, Fremont, CA, USA
ProteinChip H50 arrays	#C5730065 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip IMAC30 arrays	#C5730078 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip NP20 arrays	#C5730043 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip Q10 arrays	#C5730080 Bio-Rad Laboratories, Hercules, CA, USA
ProteinChip SELDI system enterprise edition (230 V)	Serial No. 686BR 5029 BioRad Laboratories, Fremont, CA, USA
QIAshredder (50) disposable cell lysate homogenizers	#79654 Qiagen, Hilden, Germany
Relative expression software tool (REST)-XL©-version 2	http://rest-2005.gene-quantification.info
Rotating wheel	Salmond Smith Biolab Ltd., Auckland, New Zealand
RotorGene RG-3000	Serial No. 030516 Corbett Life Science, Mortlake, NSW, Australia
Serological pipettes, stripette, 10ml, individually wrapped	#CLS4488 Sigma-Aldrich, St Louis, MO, USA
Serological pipettes, stripette, 25ml, individually wrapped	#CLS4251 Sigma-Aldrich, St Louis, MO, USA
Serological pipettes, stripette, 5ml, individually wrapped	#CLS4051 Sigma-Aldrich, St Louis, MO, USA
Sonicator, vibra cell	John Morris Scientific, NSW, Australia
Spectrophotometer, NanoDrop ND-1000	NanoDrop, Wilmington, DE, USA
SSI graduated microtubes 2ml	#1310-00 Scientific Specialties Inc., Lodi, CA, USA

StarFrost® microscope slides, Poly-L-Lysine coated	#G312P -W ProSciTech, Thuringowa, QLD, Australia
Sunrise™ microplate reader	Tecan Group Ltd., Männedorf, Switzerland
Superfrost ultra plus microscope slides	#HD41800 72P3 HD Scientific Supplies P/L, Wetherill Park BC, NSW, Australia
Thermocycler, Dyad DNA engine	Bio-Rad Laboratories, Hercules, CA, USA
TransBlot SD semi-dry transfer unit	Bio-Rad Laboratories, Hercules, CA, USA
Tube, Abgene PCR 0.5ml, domed cap	#AB0489B, AB0489G, AB0489Y Thermo Scientific, Waltham, MA, USA
Tube, centrifuge, 50ml, sterile, falcon	#352070 BD Medical, North Ryde, NSW, Australia
Tube, centrifuge, polypropylene, V-BASE 15ml,	#2323-015 Crown Scientific, Minto, NSW, Australia
Tube, cryogenic, inner thread, 1.8 ml, IWAKI	#2732-002 Crown Scientific, Minto, NSW, Australia
Tube, Real Time PCR 0.1 ml Tubes, (formerly from Corbett Research)	#981103 Qiagen, Hilden, Germany
UV transilluminator	#TM-36 UVP Inc., San Gabriel, CA, USA
Water bath	#TWB-12D Thermoline Scientific, NSW, Australia
XCELL blot module	#EI9051 Invitrogen, Carlsbad, CA, USA
XCELL surelock mini cell	#EI0001 Invitrogen, Carlsbad, CA, USA

APPENDIX B

Acetic Acid (4 M)

45.8 mL glacial acetic acid in 200ml

Acetonitrile, 50%

Add 50 ml of 100% acetonitrile to 50 ml of milliQ water.
Final volume is 100 ml.

Agar Solution for the Preparation of Cell Blocks

1.5% agar
0.85 g NaCl

1.50 agar
100 ml milliQ water

Heat in microwave until agar and NaCl well dissolved approximately 1 min and check frequently to mix as it heats up. Aliquot out into smaller volume tubes and allow to cool before storing long term at 4 °C.

Ammonium Acetate (50 mM)

Dissolve 0.3854 g of ammonium acetate in a final volume of 100ml milliQ water
Prepare 3 solutions, adjust pH of buffers to 4.0, 5.0 and 6.0
Note: Only used for optimisation

APS solution

0.1 g APS
Dissolve in 1ml milliQ water

Bromo-phenol Blue Solution (20X) 0.2%

5 mg bromo-phenol powder
Dissolve in 2.5 ml milliQ water

Blocking Solution

10 g skim milk powder→5%
Dissolve in 200 ml of 1X TBS buffer
Add 50 µl of Tween 20→0.05%

CM10 Binding Buffer (50 mM Sodium Acetate, pH 4.0)

Dissolve 0.4104 g of sodium acetate in a final volume of 100 ml milliQ water
Adjust pH to 4.0 with acetic acid

Note: 50 mM sodium acetate with pH 5.0 and pH 6.0 was used for the optimisation.

Copper Sulfate (0.1 M)

Dissolve 1.596 g in final volume of 100ml milliQ water

DNase Buffer Ambion Kit

6 µl 10X DNase Buffer
4 µl DNase
50 µl Nuclease-free Water

DTT (1M)

1.54 g of DTT
Dissolve in 10ml of milliQ water and dispense into 50 µl aliquots
Store at -20 °C.

EDTA (0.5 M) pH 8.0

186.12 g EDTA

Dissolve in final volume of 1 L milliQ water

pH adjusted to 8.0

Etoposide Phosphate

Equivalent to

100 mg etoposide

32.7 mg sodium citrate

And 30 mg dextran40

H50 Array Binding Buffer (0.1 TFA, 10% Acetonitrile)

100 µl TFA

10 ml ACN

Fill up to 100 ml with milliQ water

HEPES (50 mM), pH 7.0

Dissolve 1.19 g HEPES in 100 ml of milliQ water

Adjust pH to 7.0

IMAC Array Binding Buffer (1X PBS)

Dilute 20X PBS buffer down to 1:20 with milliQ water.

Adjust pH to 7.2

IMAC Neutralisation Buffer (0.1 M Sodium Acetate, pH 4.0)

Dissolve 1.641 g of sodium acetate in a final volume of 200 ml milliQ water

Adjust pH to 4.0 with acetic acid

MOPS (10X)

MOPS 104.6 g

Tris 60.6 g

SDS 10 g

EDTA 3 g

pH 7.7 fill up to 1000 ml

NuPAGE Transfer Buffer (20X)

10.2 g bicine

13.1 g bis-tris (free base)

0.75 g of EDTA

Dissolve in 100 ml MilliQ water and fill up to 125 ml

Adjust pH to 7.2

NuPAGE Transfer Buffer (1X) with 10% methanol

50 ml 20X NuPage transfer buffer

100 ml methanol

850 ml milliQ water

Permeabilisation Solution (TUNEL assay)

0.1% Triton X-100

0.1% sodium citrate freshly prepared in milliQ water

Preparation of EAM (Sinapinic acid)

1% TFA

100 µl TFA

In final volume of 10 ml milliQ water

20 mg of sinapinic acid

200 µl ACN

200 µl 1% TFA

Mix by vortexing for 5 minutes, briefly spin

Apply 1 µl of EAM on spots, let it dry and repeat it with again 1 µl of EAM.

Pyrosequencing Binding Buffer pH 7.6

10 mM tris 1.21 g/L
2 M NaCl 117 g/L
1 mM EDTA 0.292 g/L
0.1% Tween 1ml/L
1 M HCl to adjust pH

Pyrosequencing Wash Buffer pH 7.6

10 mM tris acetate 1.21 g/L
4 M acetic acid to adjust pH

Pyrosequencing Annealing Buffer pH 7.6

20 mM tris 2.42 g/L
2 mM MgAc₂ 0.43 g/L
4 M acetic acid to adjust pH

Pyrosequencing Denaturing Solution

0.2 M NaOH 8 g/L

Q10 Binding Buffer (50 mM Tris-HCl, pH 8.5)

Dissolve 1.21 g of tris in a final volume of 200 ml milliQ water
Adjust pH to 7.5, 8.0, 8.5 and 9.0 with HCl

Note: Three buffers with pH 7.5, 8.0, 8.5 were used for the optimisation study. pH 9.0 buffer was used for the semi-purification of protein samples.

RP-HPLC, Buffer A (0.1% TFA, 15% ACN)

1 ml of TFA
150 ml of ACN, add to milliQ water
Final volume 1 L

RP-HPLC, Buffer B (0.1%TFA, 60% ACN)

1 ml of TFA
600 ml of ACN, add to milliQ water
Final volume 1 L

Schafer-Nielson Semi-Dry Transfer Buffer

5.82 g tris→final 48 mM
2.93 g glycine→39 mM
3.75 ml 10% SDS→0.1 nM
Dissolve in 600 ml milliQ, adjust pH to 9.2
Fill up to 800 ml with milliQ water

80 ml of 1X transfer buffer mix with 20 ml of methanol
100% methanol→ 20%

Sodium acetate (50 mM) with pH 4.0, 5.0 and 6.0

Dissolve 0.4104 g of Sodim acetate in a final volume of 100 ml milliQ water.
Adjust pH of buffers to pH 4.0, 5.0 and 6.0 with acetic acid)

Washing Solution (Western Blot)

1 L 1X TBS
1 ml Tween 20

TBE Buffer (10X)

Tris base 108 g
Boric acid 55 g
0.5 EDTA (pH 8.0) 40 ml
Fill volume up to 1 L with milliQ water

Trypan Blue Solution 0.4% (w/v)

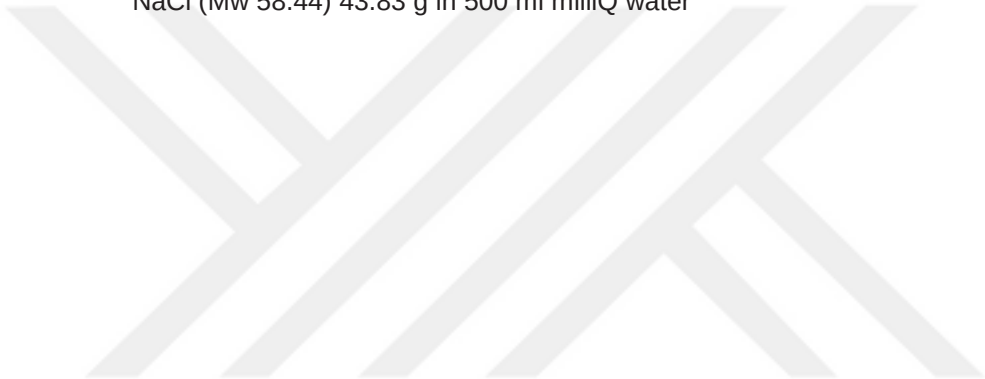
0.4 g trypan blue
Dissolve in 100 ml of 1X PBS and stored at room temperature

Tris-Glycine running buffer (10X) for Bio-Rad Mini protean TGX 4-20%

30.28 g tris 25 mM
144 g glycine 192 mM
10 g SDS 0.1%

TBS (10X)

Tris base (Mw 121.14) 12.14 g
NaCl (Mw 58.44) 43.83 g in 500 ml milliQ water



APPENDIX C

Protein Standards used in this thesis

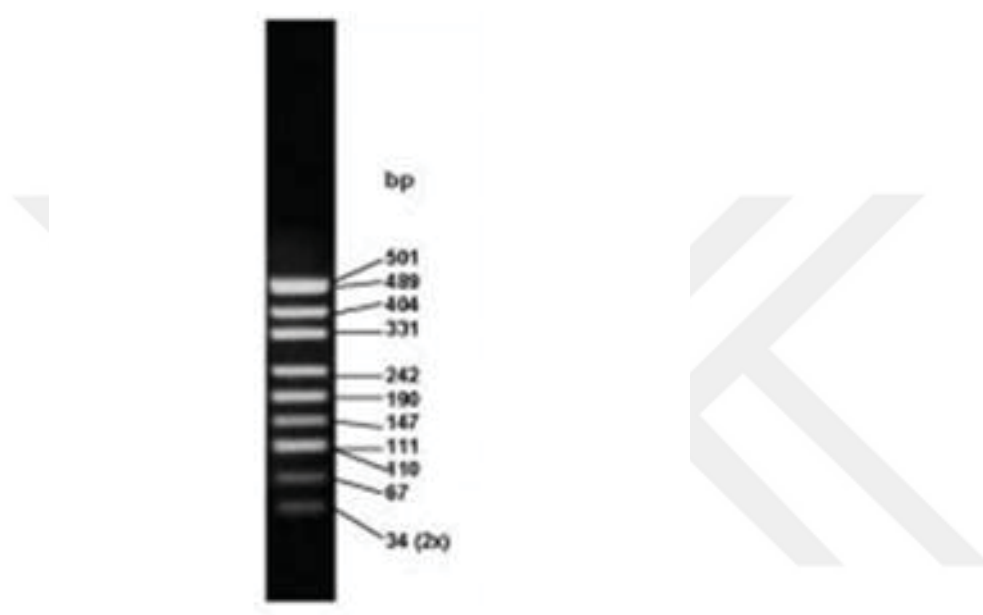


Figure C.1 DNA ladder, pUC19/HpaII

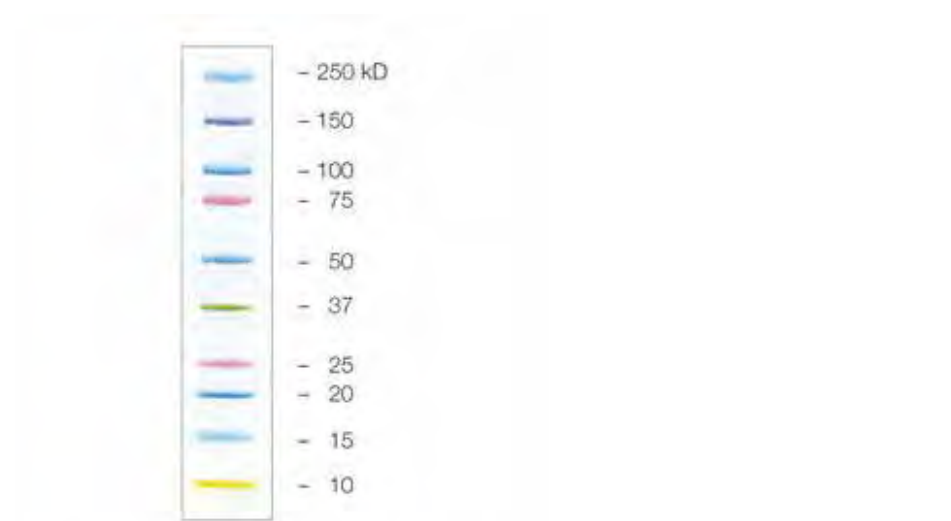


Figure C.2 Precision plus protein Kaleidoscope standards

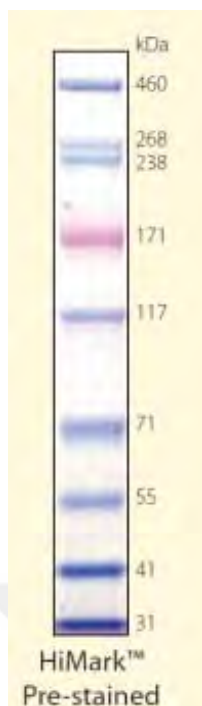


Figure C.3 HiMark* pre-stained high molecular weight protein standard

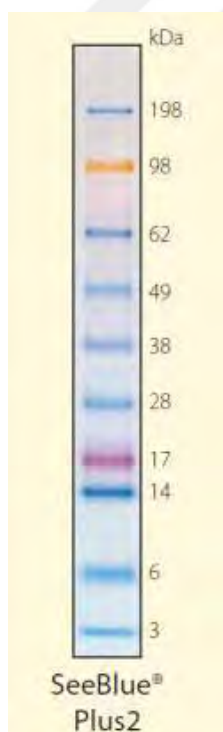


Figure C.4 SeeBlue® Plus2 pre-stained plus protein standard

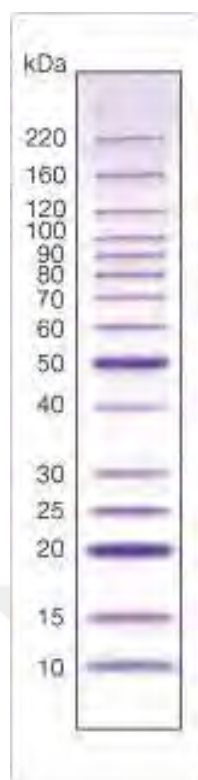


Figure C.5 BenchMark™ unstained plus, protein standard

APPENDIX D

Details of Analysis-Chapter 6

Table D.1 Up-regulated proteins in non-responders

Nr	m/z	Mean Intensity	p-value	AUC	Quality of Peaks	Result
1	10850.804h50	11.736 29.306	0.001	0.878	✓	Analysed by BLR
2	11845.022h50	2.112 4.062	0.002	0.86	✗	Eliminated
3	2914.489h50	8.759 14.565	0.004	0.828	✗	Eliminated
4	2347.671h50	6.413 10.580	0.019	0.767	✗	Eliminated
5	4439.449h50	5.437 12.183	0.01	0.794	✓	Analysed by BLR
6	9849.232h50	2.258 3.732	0.028	0.75	✗	Eliminated
7	22889.878h50	0.197 0.264	0.032	0.74	✗	Eliminated
8	12080.314h50	1.474 3.084	0.032	0.74	✗	Eliminated
9	3361.199h50	13.953 77.711	0.013	0.783	✓	Eliminated
10	12686.758h50	1.208 2.161	0.017	0.77	✗	Eliminated
11	10450.662q10	18.051 37.972	0.003	0.839	✗	Eliminated
12	10836.655q10	27.312 77.439	0.003	0.839	✓	Eliminated
13	10247.184cm10	17.531 39.693	0.001	0.889	✓	Analysed by BLR
14	12361.517cm10	11.632 22.406	0.005	0.822	✓	Analysed by BLR
15	3430.545cm10	136.327 582.470	0.011	0.789	✓	Eliminated
16	3512.901cm10	11.993 83.925	0.045	0.728	✗	Eliminated
17	13160.052cm10	1.862 5.529	0.011	0.79	✗	Eliminated
18	10433.319imac	1.911 3.980	0.025	0.76	✗	Eliminated

Table D.2 Up-regulated proteins in responders

Nr	m/z	Mean Intensity	p-value	AUC	Quality of Peaks	Result
1	7496.495h50	6.345 6.413	0.01	0.790	✖	Eliminated
2	31021.054h50	0.252 0.119	0.022	0.760	✖	Eliminated
3	15551.078h50	1.285 0.477	0.015	0.780	✖	Eliminated
4	7934.333q10	176.602 106.473	0.000	0.917	✓	Analysed by BLR
5	15865.953q10	459.882 254.388	0.002	0.856	✓	Analysed by BLR
6	16012.455q10	128.173 71.944	0.005	0.822	✓	Analysed by BLR
7	16076.491q10	142.898 77.211	0.005	0.822	✓	Analysed by BLR
8	16872.066q10	78.421 37.153	0.005	0.817	✓	Analysed by BLR
9	16129.833q10	125.376 66.813	0.005	0.817	✓	Analysed by BLR
10	17170.242q10	42.689 19.381	0.004	0.828	✓	Analysed by BLR
11	17874.649q10	17.531 6.634	0.007	0.806	✓	Analysed by BLR
12	14970.497q10	32.834 16.446	0.015	0.780	✓	Eliminated
13	31860.116q10	11.241 4.054	0.017	0.772	✖	Eliminated
14	21266.614q10	4.631 1.598	0.032	0.740	✖	Eliminated
15	5253.011q10	61.056 40.543	0.040	0.733	✓	Eliminated
16	31118.644q10	7.126 2.149	0.025	0.760	✖	Eliminated
17	4255.869q10	78.500 54.011	0.007	0.809	✓	Analysed by BLR
18	36124.841cm10	0.432 0.190	0.028	0.750	✖	Eliminated
19	20992.304imac	0.605 0.171	0.002	0.860	✖	Eliminated
20	21830.263imac	0.361 0.132	0.002	0.860	✖	Eliminated
21	14967.406imac	20.588 4.790	0.013	0.783	✓	Eliminated
22	15773.177imac	17.633 8.387	0.028	0.750	✖	Eliminated
23	20189.254imac	2.935 0.774	0.028	0.750	✖	Eliminated
24	7931.295imac	55.349 29.007	0.045	0.728	✓	Eliminated
25	5151.826imac	51.691 28.583	0.036	0.739	✓	Eliminated
26	15128.829imac	233.443 110.432	0.040	0.733	✖	Eliminated

Table D.3 Up-regulated proteins in STS

Nr	m/z	Mean intensity	p-value	AUC	Quality of Peaks	Result
1	2084.723h50	24.622 18.971	0.026	0.712	✗	Eliminated
2	3678.923q10	61.772 27.992	0.005	0.765	✓	Analysed by BLR
3	12076.159cm10	4.995 0.764	0.001	0.823	✗	Eliminated
4	11957.837cm10	6.063 2.156	0.001	0.820	✗	Eliminated
5	11649.702cm10	23.269 10.385	0.002	0.789	✗	Eliminated
6	12398.433cm10	16.022 9.175	0.005	0.767	✓	Analysed by BLR
7	12503.026cm10	8.159 4.662	0.005	0.767	✗	Eliminated
8	11632.896cm10	16.563 7.274	0.005	0.765	✗	Eliminated
9	11658.563cm10	32.563 13.550	0.007	0.756	✗	Eliminated
10	12262.571cm10	5.486 2.537	0.006	0.762	✗	Eliminated
11	3564.627cm10	32.682 17.552	0.002	0.798	✗	Eliminated
12	3660.391cm10	84.412 41.393	0.008	0.753	✗	Eliminated
13	12227.244cm10	8.083 3.751	0.012	0.745	✗	Eliminated
14	3579.500cm10	92.894 46.197	0.012	0.740	✓	Eliminated
15	11560.080cm10	24.286 9.906	0.014	0.734	✓	Analysed by BLR
16	27372.269cm10	1.157 0.555	0.014	0.734	✗	Eliminated
17	11796.596cm10	12.239 6.444	0.015	0.731	✗	Eliminated
18	11608.014cm10	21.097 9.176	0.024	0.715	✓	Analysed by BLR
19	6074.044cm10	9.721 6.169	0.022	0.717	✗	Eliminated
20	11589.354cm10	14.678 6.333	0.022	0.717	✗	Eliminated
21	19727.308cm10	1.159 0.851	0.026	0.717	✗	Eliminated
22	4142.434cm10	20.188 14.556	0.009	0.748	✗	Eliminated
23	19534.724cm10	2.000 1.246	0.026	0.712	✗	Eliminated
24	11672.275cm10	28.886 15.695	0.034	0.701	✓	Eliminated
25	3381.962cm10	98.569 53.543	0.042	0.693	✗	Eliminated
26	12185.827cm10	30.503 13.404	0.032	0.704	✓	Eliminated
27	3517.570cm10	31.304 16.012	0.012	0.740	✗	Eliminated
28	3015.767cm10	4.886 3.878	0.040	0.695	✗	Eliminated
29	12203.612cm10	13.965 6.708	0.046	0.690	✗	Eliminated
30	3536.015cm10	77.765 33.469	0.049	0.687	✓	Eliminated
31	1564.942imac	14.348 9.216	0.008	0.753	✗	Eliminated
32	4640.222imac	30.905 12.792	0.008	0.751	✗	Eliminated

Table D.4 Up-regulated proteins in LTS

Nr	m/z	Mean intensity	p-value	AUC	Quality of Peaks	Result
1	7934.333q10	127.725 162.431	0.015	0.731	✓	Analysed by BLR
2	15866.337q10	328.951 429.194	0.015	0.731	✓	Analysed by BLR
3	5590.178cm10	12.435 23.816	0.028	0.709	✗	Eliminated
4	5306.221cm10	16.729 30.559	0.032	0.704	✗	Eliminated
5	5151.445imac	25.021 46.224	0.002	0.798	✗	Eliminated
6	2571.479imac	0.591 16.585	0.008	0.753	✗	Eliminated
7	5191.461imac	117.663 225.405	0.020	0.720	✓	Analysed by BLR

APPENDIX E

NORTHERN SYDNEY
CENTRAL COAST
NSW HEALTH

19th October 2009

Dr K McDonald
Kolling Institute of Medical Research
Cancer Genetics Unit
St Leonards NSW 2065

Dear Dr K McDonald,

Re: Protocol 0911-303M(Tissue Bank) - K McDonald, H Sevim, R Baxter
Proteomic profiling of glioblastoma multiforme: Using protein signatures to
define pateints with a favourable outcome and to identify protein targets for
therapeutic intervention.

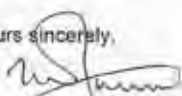
The HAWKESBURY Human Research Ethics Committee (HREC) of Northern Sydney Central Coast Health (NSCCH), has reviewed your **Tissue Bank Access Request** and I am pleased to inform you that approval has been given for you to proceed with your study.

The study has been assigned a protocol number (**0911-303M**) and we ask you to quote this number whenever you contact this office about matters relating to the study.

In order to comply with the Guidelines for Good Clinical Research Practice (GCRP) in Australia, and in line with NSCCH HREC policy, may I remind you that it is the chief investigator's responsibility to ensure that:

1. A report is provided to the HREC at the completion of the study.
2. The HREC is notified as soon as possible of any changes to the protocol. All changes must be approved by the HREC before continuation of the research project. This includes notifying the HREC of any changes to the staff involved with the protocol.

Yours sincerely,



Professor Stewart Dunn
Chairperson
HAWKESBURY HREC
NORTHERN SYDNEY
CENTRAL COAST HEALTH

RESEARCH BUSINESS UNIT
Level 2, Building 51, Royal North Shore Hospital, Pacific Hwy, ST Leonards NSW 2065
Telephone 02 9926 8106; Facsimile 02 9926 6179

11 May, 2010

Dr K McDonald
Kolling Institute of Medical Research
Cancer Genetics Unit
St Leonards NSW 2065

Dear Dr McDonald,

Re: Protocol 0911-303M (Tissue Bank) - K McDonald, H Sevim, R Baxter
Proteomic profiling of glioblastoma multiforme: Using protein signatures to define patients with a favourable outcome and to identify protein targets for therapeutic intervention.

Thank you for your application dated 3 May 2010 (received 10 May 2010), requesting approval for an amendment from the HAWKESBURY Human Research Ethics Committee (HREC) of Northern Sydney Central Coast Health (NSCCH). I am pleased to inform you that your amendment for the protocol on the above study has now been approved.

Description of amendment:

- The original application requested fresh frozen tissue for protein extraction. We hereby request additional 2x10 µm ribbons of FFPE tissue from the same patients.

The updated amendment involves the following documentation:

- Amendment Form dated 3 May 2010.
- Change of Address for Dr Kerrie McDonald.

The HREC recommends that you consult with your Medical Defence Union to ensure that you are adequately covered for the purpose of conducting this clinical trial.

In order to comply with the *Guidelines For Good Clinical Research Practice (GCRP) in Australia*, and in line with NSCCH HREC policy, may I remind you that it is the Chief Investigator's responsibility and a condition of approval, to ensure that:

1. The HREC is notified of anything that might warrant review of the ethical approval of the project, including unforeseen events that might affect the ethical acceptability of the project.
2. The HREC is notified of all Serious Adverse Events (SAEs) or Serious Unexpected Suspected Adverse Reactions (SUSARs) in accordance with the Serious Adverse Event Reporting Guidelines. Please refer to the Research Office website.
3. Proposed amendments to the research protocol or conduct of the research that may affect the ethical acceptability of the project are submitted to the HREC on an amendment form (including any relevant attachments). For multi-centre studies, the Chief Investigator should submit to the Lead HREC and then send the amendment approval letter to the investigators at each of the sites so that they can notify their Research Governance Officer.

RESEARCH BUSINESS UNIT
Level 2, Building 51, Royal North Shore Hospital, Pacific Hwy, ST Leonards NSW 2065
Telephone 02 9926 8106; Facsimile 02 9926 6179

NORTHERN SYDNEY
CENTRAL COAST
NSW HEALTH

4. Proposed changes to the personnel involved in the study are submitted to the HREC on a Change in Personnel Form (accompanied by the investigator's CV where applicable).
5. The HREC must be provided with an annual progress report for the study by the 31st October each year. For multi-centre studies the Chief Investigator should submit to the Lead HREC on behalf of all sites.
6. The HREC must also be provided with a final report upon completion of the study. For multi-centre studies the Chief Investigator should notify the Lead HREC and the investigators at each site should notify the relevant Research Governance Officer.
7. The HREC must be notified, giving reasons if the project is discontinued at a site before the expected date of completion.

Please refer to the NSCCAHS Research Office website to access forms such as the amendment form, Annual/Final Report Form, Change in Personnel Form and Serious Adverse Event Guidelines and Forms;

Intranet:

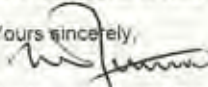
<http://intranet01.nsaahs.nsw.gov.au/intranet/rns/research/Ethics/HREC/default.shtml>

Internet:

<http://www.nscchhs.health.nsw.gov.au/services/research/default.shtml>

HREC approval is valid for four (4) years from the date of the approval letter.

Yours sincerely,



Professor Stewart Dunn
Chairperson
HAWKESBURY HREC
NORTHERN SYDNEY
CENTRAL COAST HEALTH

RESEARCH BUSINESS UNIT

Level 2, Building 51, Royal North Shore Hospital, Pacific Hwy, ST Leonards NSW 2065
Telephone 02 9926 8106; Facsimile 02 9926 8179

11 May, 2010

Dr K McDonald
Kolling Institute of Medical Research
Cancer Genetics Unit
St Leonards NSW 2065

Dear Dr McDonald,

Re: Protocol 0911-303M(Tissue Bank) - K McDonald, H Sevim, R Baxter
Proteomic profiling of glioblastoma multiforme: Using protein signatures to
define patients with a favourable outcome and to identify protein targets for
therapeutic intervention.

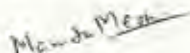
I can confirm that the Northern Sydney Central Coast Health Human Research Ethics
Committee is constituted and functions in full compliance with NHMRC Guidelines.

Currently, there are nineteen (19) members of this Committee including:

- Laywomen
- Laymen
- a Minister of Religion
- 2 Lawyers
- 5 Medical Graduates
- a Scientist with research experience
- a Clinical Trials Pharmacist
- an alternate Clinical Trials Pharmacist
- a Neuropsychologist
- a Psychologist
- Registered Nurses

It is not the policy of this organisation to provide the names of committee members.

Yours sincerely



Mamta Merai
Ethics Officer
HAWKESBURY HREC
Research Office
NORTHERN SYDNEY CENTRAL COAST HEALTH

RESEARCH BUSINESS UNIT
Level 2, Building 51, Royal North Shore Hospital, Pacific Hwy, ST Leonards NSW 2065
Telephone 02 9926 8106; Facsimile 02 9926 6179

REFERENCES

- Abe T., Mori T., Wakabayashi Y., Nakagawa M., Cole S.P.C., Koike K., Kuwano M., Hori S. (1998) Expression of multidrug resistance protein gene in patients with glioma after chemotherapy. *Journal of Neuro-Oncology* 40:11-18.
- Abe T., Hasegawa S., Taniguchi K., Yokomizo A., Kuwano T., Ono M., Mori T., Hori S., Kohno K., Kuwano M. (1994) Possible Involvement of Multidrug-Resistance-Associated Protein (Mrp) Gene-Expression in Spontaneous Drug-Resistance to Vincristine, Etoposide and Adriamycin in Human Glioma-Cells. *International Journal of Cancer* 58:860-864.
- Adam B.L., Qu Y.S., Davis J.W., Ward M.D., Clements M.A., Cazares L.H., Semmes O.J., Schellhammer P.F., Yasui Y., Feng Z.D., Wright G.L. (2002) Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Research* 62:3609-3614.
- Aebersold R., Goodlett D.R. (2001) Mass spectrometry in proteomics. *Chemical Reviews* 101:269-295. DOI: Doi 10.1021/Cr990076h.
- Agarwal M.L., Agarwal A., Taylor W.R., Stark G.R. (1995) P53 Controls Both the G(2)/M and the G(1) Cell-Cycle Checkpoints and Mediates Reversible Growth Arrest in Human Fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* 92:8493-8497.
- Agarwal S., Sane R., Oberoi R., Ohlfest J.R., Elmquist W.F. (2011) Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. *Expert Reviews in Molecular Medicine* 13. DOI: ARTN e17 DOI 10.1017/S1462399411001888.
- AIHW. (2010) Cancer in Australia: an overview, 2010. Australian Institute of Health and Welfare (AIHW) & Australasian Association of Cancer Registries 2010 Cancer series no. 60. Cat. no. CAN 56. Canberra: AIHW.
- Al-Mayhany M.T.F., Grenfell R., Narita M., Piccirillo S., Kenney-Herbert E., Fawcett J.W., Collins V.P., Ichimura K., Watts C. (2011) NG2 expression in glioblastoma identifies an actively proliferating population with an aggressive molecular signature. *Neuro-Oncology* 13:830-845. DOI: DOI 10.1093/neuonc/nor088.
- Al-Tarawneh S.K., Bencharit S. (2009) Applications of Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight (SELDI-TOF) Mass Spectrometry in Defining Salivary Proteomic Profiles. *Open Dent J* 3:74-79. DOI: 10.2174/1874210600903010074.
- Albrethsen J., Moller C.H., Olsen J., Raskov H., Gammeltoft S. (2006) Human neutrophil peptides 1, 2 and 3 are biochemical markers for metastatic colorectal cancer. *European Journal of Cancer* 42:3057-3064. DOI: DOI 10.1016/j.ejca.2006.05.039.
- Alghisi G.C., Ponsonnet L., Ruegg C. (2009) The Integrin Antagonist Cilengitide Activates alpha V beta 3, Disrupts VE-Cadherin Localization at Cell Junctions and Enhances Permeability in Endothelial Cells. *Plos One* 4. DOI: Artin E4449 Doi 10.1371/Journal.Pone.0004449.

- Allalunis-turner M.J., Barron G.M., Day R.S., Dobler K.D., Mirzayans R. (1993) Isolation of 2 Cell-Lines from a Human-Malignant Glioma Specimen Differing in Sensitivity to Radiation and Chemotherapeutic Drugs. *Radiation Research* 134:349-354.
- Allard L., Lescuyer P., Burgess J., Leung K.Y., Ward M., Walter N., Burkhard P.R., Corthals G., Hochstrasser D.F., Sanchez J.C. (2004) ApoC-I and ApoC-III as potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke. *Proteomics* 4:2242-51. DOI: 10.1002/pmic.200300809.
- Allen L.A., Felker G.M. (2010) Multi-marker strategies in heart failure: clinical and statistical approaches. *Heart Failure Reviews* 15:343-349. DOI: DOI 10.1007/s10741-009-9144-z.
- Allred D.C., Harvey J.M., Berardo M., Clark G.M. (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 11:155-68.
- Amin M.A., Volpert O.V., Woods J.M., Kumar P., Harlow L.A., Koch A.E. (2003) Migration inhibitory factor mediates angiogenesis via mitogen-activated protein kinase and phosphatidylinositol kinase. *Circulation Research* 93:321-329. DOI: Doi 10.1161/01.Res.0000087641.56024.Da.
- Arai K., Teratani T., Nozawa R., Yamada T. (2001) Immunohistochemical investigation of S100A9 expression in pulmonary adenocarcinoma: S100A9 expression is associated with tumor differentiation. *Oncology Reports* 8:591-596.
- Asada N., Tsuchiya H., Tomita K. (1999) De novo deletions of p53 gene and wild-type p53 correlate with acquired cisplatin-resistance in human osteosarcoma OST cell line. *Anticancer Res* 19:5131-7.
- Asano T., An T.H., Mayes J., Zwelling L.A., Kleiner E.S. (1996) Transfection of human topoisomerase II alpha into etoposide-resistant cells: Transient increase in sensitivity followed by down-regulation of the endogenous gene. *Biochemical Journal* 319:307-313.
- Ashby L.S., Shapiro W.R. (2001) Intra-arterial cisplatin plus oral etoposide for the treatment of recurrent malignant glioma: a phase II study. *Journal of Neuro-Oncology* 51:67-86.
- Bacher M., Schrader J., Thompson N., Kuschela K., Gerns D., Waeber G., Schlegel J. (2003) Up-regulation of macrophage migration inhibitory factor gene and protein expression in glial tumor cells during hypoxic and hypoglycemic stress indicates a critical role for angiogenesis in glioblastoma multiforme. *American Journal of Pathology* 162:11-17.
- Bacher M., Metz C.N., Calandra T., Mayer K., Chesney J., Lohoff M., Gerns D., Donnelly T., Bucala R. (1996) An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. *Proceedings of the National Academy of Sciences of the United States of America* 93:7849-7854.
- Baillet A., Trocme C., Berthier S., Arlotto M., Grange L., Chenau J., Quetant S., Seve M., Berger F., Juvin R., Morel F., Gaudin P. (2010) Synovial fluid proteomic fingerprint: S100A8, S100A9 and S100A12 proteins discriminate rheumatoid arthritis from other inflammatory joint diseases. *Rheumatology* 49:671-682. DOI: DOI 10.1093/rheumatology/kep452.

- Baines C.J., Miller A.B., Wall C., Mcfarlane D.V., Simor I.S., Jong R., Shapiro B.J., Audet L., Petitclerc M., Ouimetoliva D., Ladouceur J., Hebert G., Minuk T., Hardy G., Standing H.K. (1986) Sensitivity and Specificity of 1st Screen Mammography in the Canadian National Breast Screening Study - a Preliminary-Report from 5 Centers. *Radiology* 160:295-298.
- Baird C.L., Harkins T.T., Morris S.K., Lindsley J.E. (1999) Topoisomerase II drives DNA transport by hydrolyzing one ATP. *Proceedings of the National Academy of Sciences of the United States of America* 96:13685-13690.
- Baker M. (2005) In biomarkers we trust? *Nature Biotechnology* 23:297-304.
- Balestrieri M.L., Giovane A., Mancini F.P., Napoli C. (2008) Proteomics and cardiovascular disease: An update. *Current Medicinal Chemistry* 15:555-572.
- Balss J., Meyer J., Mueller W., Korshunov A., Hartmann C., von Deimling A. (2008) Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathologica* 116:597-602. DOI: DOI 10.1007/s00401-008-0455-2.
- Bando H., Matsumoto G., Bando M., Muta M., Ogawa T., Funata N., Nishihira J., Koike M., Toi M. (2002) Expression of macrophage migration inhibitory factor in human breast cancer: Association with nodal spread. *Japanese Journal of Cancer Research* 93:389-396.
- Bandtlow C.E., Zimmermann D.R. (2000) Proteoglycans in the developing brain: New conceptual insights for old proteins. *Physiological Reviews* 80:1267-1290.
- Banez L.L., Prasanna P., Sun L., Ali A., Zou Z.Q., McLeod D.G., Moul J.W., Srivastava S. (2003) Diagnostic potential of serum proteomic patterns in prostate cancer. *Journal of Urology* 169:54-55.
- Banks R.E., Dunn M.J., Hochstrasser D.F., Sanchez J.C., Blackstock W., Pappin D.J., Selby P.J. (2000) Proteomics: new perspectives, new biomedical opportunities. *Lancet* 356:1749-1756.
- Bao S.D., Wu Q.L., Sathornsumetee S., Hao Y.L., Li Z.Z., Hjelmeland A.B., Shi O., McLendon R.E., Bigner D.D., Rich J.N. (2006) Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Research* 66:7843-7848. DOI: Doi 10.1158/0008-5472.Can-06-1010.
- Baracska K.L., Kidd G.J., Miller R.H., Trapp B.D. (2007) NG2-positive cells generate A2B5-positive oligodendrocyte precursor cells. *Glia* 55:1001-1010. DOI: Doi 10.1002/Glia.20519.
- Baron N., Deuster O., Noelker C., Stuer C., Strik H., Schaller C., Dodel R., Meyer B., Bacher M. (2011) Role of macrophage migration inhibitory factor in primary glioblastoma multiforme cells. *J Neurosci Res* 89:711-7. DOI: 10.1002/jnr.22595.
- Barritt D.S., Pearn M.T., Zisch A.H., Lee S.S., Javier R.T., Pasquale E.B., Stallcup W.B. (2000) The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. *Journal of Cellular Biochemistry* 79:213-224.
- Barthelmes H.U., Habermeyer M., Christensen M.O., Mielke C., Interthal H., Pouliot J.J., Boege F., Marko D. (2004) TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. *Journal of Biological Chemistry* 279:55618-55625. DOI: 10.1074/jbc.M405042200.

- Bast R.C., Feeney M., Lazarus H., Nadler L.M., Colvin R.B., Knapp R.C. (1981) Reactivity of a Monoclonal-Antibody with Human Ovarian-Carcinoma. *Journal of Clinical Investigation* 68:1331-1337.
- Bast R.C., Jr. (2003) Status of tumor markers in ovarian cancer screening. *J Clin Oncol* 21:200s-205s. DOI: 10.1200/JCO.2003.01.068
- JCO.2003.01.068 [pii].Bauer R., Ratzinger S., Wales L., Bosserhoff A., Senner V., Grifka J., Grassel S. (2011) Inhibition of Collagen XVI Expression Reduces Glioma Cell Invasiveness. *Cellular Physiology and Biochemistry* 27:217-226.
- Baugh J.A., Gantier M., Li L., Byrne A., Buckley A., Donnelly S.C. (2006) Dual regulation of macrophage migration inhibitory factor (MIF) expression in hypoxia by CREB and HIF-1. *Biochemical and Biophysical Research Communications* 347:895-903. DOI: DOI 10.1016/j.bbrc.2006.06.148.
- Beauchesne P., Bernier V., Carnin C., Taillandier L., Djabri M., Martin L., Michel X., Maire J.P., Khalil T., Kerr C., Gorlia T., Stupp R., Pedoux R. (2010) Prolonged survival for patients with newly diagnosed, inoperable glioblastoma with 3-times daily ultrafractionated radiation therapy. *Neuro-Oncology* 12:595-602. DOI: DOI 10.1093/neuonc/noq008.
- Behm F.G., Smith F.O., Raimondi S.C., Pui C.H., Bernstein I.D. (1996) Human homologue of the rat chondroitin sulfate proteoglycan, NG2, detected by monoclonal antibody 7.1, identifies childhood acute lymphoblastic leukemias with t(4;11)(q21;q23) or t(11;19)(q23;p13) and MLL gene rearrangements. *Blood* 87:1134-1139.
- Benassi M.S., Pazzaglia L., Chiechi A., Alberghini M., Conti A., Cattaruzza S., Wassermann B., Picci P., Perris R. (2009) NG2 Expression Predicts the Metastasis Formation in Soft-Tissue Sarcoma Patients. *Journal of Orthopaedic Research* 27:135-140. DOI: Doi 10.1002/Jor.20694.
- Bennett L., Palucka A.K., Arce E., Cantrell V., Borvak J., Banchereau J., Pascual V. (2003) Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *Journal of Experimental Medicine* 197:711-723. DOI: Doi 10.1084/Jem.20021553.
- Berger J.M., Gamblin S.J., Harrison S.C., Wang J.C. (1996) Structure and mechanism of DNA topoisomerase II.[erratum appears in *Nature* 1996 Mar 14;380(6570):179]. *Nature* 379:225-32.
- Berkenkamp S., Karas M., Hillenkamp F. (1996) Ice as a matrix for IR-matrix-assisted laser desorption/ionization: Mass spectra from a protein single crystal. *Proceedings of the National Academy of Sciences of the United States of America* 93:7003-7007.
- Berns E.M., Foekens J.A., Vossen R., Look M.P., Devilee P., Henzen-Logmans S.C., van Staveren I.L., van Putten W.L., Inganas M., Meijer-van Gelder M.E., Cornelisse C., Claassen C.J., Portengen H., Bakker B., Klijn J.G. (2000) Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. *Cancer Res* 60:2155-62.

- Bianco R.B., Melisi D., Ciardiello F., Tortora G. (2006) Key cancer cell signal transduction pathways as therapeutic targets. *European Journal of Cancer* 42:290-294. DOI: DOI 10.1016/j.ejca.2005.07.034.
- Blakeley J. (2008) Drug delivery to brain tumors. *Current Neurology and Neuroscience Reports* 8:235-241.
- Board R., Jayson G.C. (2005) Platelet-derived growth factor receptor (PDGFR): A target for anticancer therapeutics. *Drug Resistance Updates* 8:75-83. DOI: DOI 10.1016/j.drug.2005.03.004.
- Bode G., Luken A., Kerkhoff C., Roth J., Ludwig S., Nacken W. (2008) Interaction between S100A8/A9 and Annexin A6 Is Involved in the Calcium-induced Cell Surface Exposition of S100A8/A9. *Journal of Biological Chemistry* 283:31776-31784. DOI: DOI 10.1074/jbc.M803908200.
- Boja E.S., Fales H.M. (2001) Overalkylation of a protein digest with iodoacetamide. *Analytical Chemistry* 73:3576-3582.
- Bouamrani A., Ternier J., Ratel D., Benabid A.L., Issartel J.P., Brambilla E., Berger F. (2006a) Direct-tissue SELDI-TOF mass spectrometry analysis: a new application for clinical proteomics. *Clinical Chemistry* 52:2103-6. DOI: clinchem.2006.070979 [pii] 10.1373/clinchem.2006.070979.
- Bouamrani A., Ternier J., Ratel D., Benabid A.L., Issartel J.P., Brambilla E., Berger F., Bouamrani A., Ternier J., Ratel D., Benabid A.-L., Issartel J.-P., Brambilla E., Berger F. (2006b) Direct-tissue SELDI-TOF mass spectrometry analysis: a new application for clinical proteomics.[see comment]. *Clinical Chemistry* 52:2103-6.
- Bourne T.D., Schiff D. (2010) Update on molecular findings, management and outcome in low-grade gliomas. *Nature Reviews Neurology* 6:695-701. DOI: DOI 10.1038/nrneurol.2010.159.
- Bower M., Newlands E.S., Bleehen N.M., Brada M., Begent R.J.H., Calvert H., Colquhoun I., Lewis P., Brampton M.H. (1997) Multicentre CRC phase II trial of temozolomide in recurrent or progressive high-grade glioma. *Cancer Chemotherapy and Pharmacology* 40:484-488.
- Brandes A.A., Ermani M., Basso U., Amista P., Berti F., Scienza R., Rotilio A., Pinna G., Gardiman M., Monfardini S. (2001) Temozolomide as a second-line systemic regimen in recurrent high-grade glioma: A phase II study. *Annals of Oncology* 12:255-257.
- Brandes A.A., Tosoni A., Cavallo G., Reni M., Franceschi E., Bonaldi L., Bertorelle R., Gardiman M., Ghimenton C., Iuzzolino P., Pession A., Blatt V., Ermani M. (2006) Correlations between O6-methylguanine DNA methyltransferase promoter methylation status, 1p and 19q deletions, and response to temozolomide in anaplastic and recurrent oligodendroglioma: A prospective GICNO study. *Journal of Clinical Oncology* 24:4746-4753. DOI: Doi 10.1200/Jco.2006.06.3891.

- Brat D.J., Castellano-Sanchez A.A., Hunter S.B., Pecot M., Cohen C., Hammond E.H., Devi S.N., Kaur B., Van Meir E.G. (2004) Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. *Cancer Research* 64:920-927.
- Bredel M., Zentner J., Bredel M., Zentner J. (2002a) Brain-tumour drug resistance: the bare essentials. *Lancet Oncology* 3:397-406.
- Bredel M., Piribauer M., Marosi C., Birner P., Gatterbauer B., Fischer I., Strobel T., Rossler K., Budka H., Hainfellner J.A., Bredel M., Piribauer M., Marosi C., Birner P., Gatterbauer B., Fischer I., Strobel T., Rossler K., Budka H., Hainfellner J.A. (2002b) High expression of DNA topoisomerase IIalpha and Ki-67 antigen is associated with prolonged survival in glioblastoma patients. *European Journal of Cancer* 38:1343-7.
- Brell M., Ibanez J., Tortosa A. (2011) O6-Methylguanine-DNA methyltransferase protein expression by immunohistochemistry in brain and non-brain systemic tumours: systematic review and meta-analysis of correlation with methylation-specific polymerase chain reaction. *Bmc Cancer* 11. DOI: Artn 35 Doi 10.1186/1471-2407-11-35.
- Brell M., Tortosa A., Verger E., Gil J.M., Vinolas N., Villa S., Acebes J.J., Caral L., Pujol T., Ferrer I., Ribalta T., Graus F. (2005) Prognostic significance of O-6-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clinical Cancer Research* 11:5167-5174.
- Briancon-Marjollet A., Balenci L., Fernandez M., Esteve F., Honnorat J., Farion R., Beaumont M., Barbier E., Remy C., Baudier J. (2010) NG2-expressing glial precursor cells are a new potential oligodendroglioma cell initiating population in N-ethyl-N-nitrosourea-induced gliomagenesis. *Carcinogenesis*. DOI: bgq154 [pii] 10.1093/carcin/bgq154.
- Bromberg J.E.C., van den Bent M.J. (2009) Oligodendrogliomas: Molecular Biology and Treatment. *Oncologist* 14:155-163. DOI: DOI 10.1634/theoncologist.2008-0248.
- Bromberg K.D., Osheroff N. (2001) DNA cleavage and religation by human topoisomerase II alpha at high temperature. *Biochemistry* 40:8410-8.
- Bromberg K.D., Burgin A.B., Osheroff N., Bromberg K.D., Burgin A.B., Osheroff N. (2003) A two-drug model for etoposide action against human topoisomerase IIalpha. *Journal of Biological Chemistry* 278:7406-12.
- Bromberg K.D., Velez-Cruz R., Burgin A.B., Osheroff N., Bromberg K.D., Velez-Cruz R., Burgin A.B., Osheroff N. (2004) DNA ligation catalyzed by human topoisomerase II alpha. *Biochemistry* 43:13416-23.
- Buonerba C., Di Lorenzo G., Marinelli A., Federico P., Palmieri G., Imbimbo M., Conti P., Peluso G., De Placido S., Sampson J.H. (2011) A comprehensive outlook on

- Burg M.A., Grako K.A., Stallcup W.B. (1998) Expression of the NG2 proteoglycan enhances the growth and metastatic properties of melanoma cells. *Journal of Cellular Physiology* 177:299-312.
- Burg M.A., Tillet E., Timpl R., Stallcup W.B. (1996) Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. *Journal of Biological Chemistry* 271:26110-26116.
- Burg M.A., Pasqualini R., Arap W., Ruoslahti E., Stallcup W.B. (1999) NG2 proteoglycan-binding peptides target tumor neovasculature. *Cancer Research* 59:2869-2874.
- Burger P.C., Kleihues P. (1989) Cytologic Composition of the Untreated Glioblastoma with Implications for Evaluation of Needle Biopsies. *Cancer* 63:2014-2023.
- Burnet N.G., Jefferies S.J., Benson R.J., Hunt D.P., Treasure F.P. (2005) Years of life lost (YLL) from cancer is an important measure of population burden--and should be considered when allocating research funds. *British Journal of Cancer* 92:241-5.
- Butterfield D.A., Boyd-Kimball D., Castegna A. (2003) Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. *Journal of Neurochemistry* 86:1313-1327. DOI: DOI 10.1046/j.1471.2003.01948.x.
- Cairncross G., Macdonald D., Ludwin S., Lee D., Cascino T., Buckner J., Fulton D., Dropcho E., Stewart D., Schold C., Wainman N., Eisenhauer E. (1994) Chemotherapy for Anaplastic Oligodendroglioma. *Journal of Clinical Oncology* 12:2013-2021.
- Cairncross J.G., Ueki K., Zlatescu M.C., Lisle D.K., Finkelstein D.M., Hammond R.R., Silver J.S., Stark P.C., Macdonald D.R., Ino Y., Ramsay D.A., Louis D.N. (1998) Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *Journal of the National Cancer Institute* 90:1473-1479.
- Calandra T., Roger T. (2003) Macrophage migration inhibitory factor: A regulator of innate immunity. *Nature Reviews Immunology* 3:791-800. DOI: Doi 10.1038/Nri1200.
- Calandra T., Bernhagen J., Mitchell R.A., Bucala R. (1994) Macrophage Is an Important and Previously Unrecognized Source of Macrophage-Migration Inhibitory Factor. *Journal of Experimental Medicine* 179:1895-1902.
- Calandra T., Bernhagen J., Metz C.N., Spiegel L.A., Bacher M., Donnelly T., Cerami A., Bucala R. (1995) Mif as a Glucocorticoid-Induced Modulator of Cytokine Production. *Nature* 377:68-71.
- Calogero A., Porcellini A., Lombardi V., Fabbiano C., Arcella A., Miscusi M., Ponti D., Ragona G. (2011) Sensitivity to cisplatin in primary cell lines derived from human glioma correlates with levels of EGR-1 expression. *Cancer Cell International* 11. DOI: Artn 5 Doi 10.1186/1475-2867-11-5.
- Cao V.T., Lung T.Y., Jung S., Jin S.G., Moon K.S., Kim I.Y., Kang S.S., Park C.S., Lee K.H., Chae H.J. (2009) The Correlation and Prognostic Significance of Mgmt Promoter Methylation and Mgmt Protein in Glioblastomas. *Neurosurgery* 65:866-875. DOI: Doi 10.1227/01.Neu.0000357325.90347.A1.

- Caputo E., Lombardi M.L., Luongo V., Moharram R., Tornatore P., Pirozzi G., Guardiola J., Martin B.A. (2005) Peptide profiling in epithelial tumor plasma by the emerging proteomic techniques. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 819:59-66. DOI: DOI 10.1016/j.jchromb.2005.01.022.
- Carapella C.M., Telera S., Oppido P.A. (2011) Surgery of malignant gliomas: advances and perspectives. *Current Opinion in Oncology* 23:624-629. DOI: Doi 10.1097/Cco.0b013e32834ace58.
- Carrette O., Burkhard P.R., Sanchez J.C., Hochstrasser D.F. (2006) State-of-the-art two-dimensional gel electrophoresis: a key tool of proteomics research. *Nature Protocols* 1:812-823. DOI: DOI 10.1038/nprot.2006.117.
- Cattaruzza S., Nicolosi P.A., Perris R. (2008) Proteoglycans in the control of tumor growth and metastasis formation. *Connective Tissue Research* 49:225-229. DOI: Doi 10.1080/03008200802143448.
- CCA. (2011) Adult gliomas (astrocytomas and oligodendrogliomas): a guide for patients, their families and carers. Cancer Council Australia (CCA) / Clinical Oncological Society of Australia; 2011.
- Cech N.B., Enke C.G. (2000) Relating electrospray ionization response to nonpolar character of small peptides. *Analytical Chemistry* 72:2717-2723. DOI: Doi 10.1021/Ac9914869.
- Ceppi P., Longo M., Volante M., Novello S., Cappia S., Bacillo E., Selvaggi G., Saviozzi S., Calogero R., Papotti M., Scagliotti G.V. (2008) Excision repair cross complementing-1 and topoisomerase IIalpha gene expression in small-cell lung cancer patients treated with platinum and etoposide: a retrospective study. *J Thorac Oncol* 3:583-9. DOI: 10.1097/JTO.0b013e3181734f24 01243894-200806000-00005 [pii].
- Chalkley R.J., Baker P.R., Medzihradszky K.F., Lynn A.J., Burlingame A.L. (2008) In-depth Analysis of Tandem Mass Spectrometry Data from Disparate Instrument Types. *Molecular & Cellular Proteomics* 7:2386-2398. DOI: DOI 10.1074/mcp.M800021-MCP200.
- Chaly Y.V., Paleolog E.M., Kolesnikova T.S., Tikhonov I.I., Petratchenko E.V., Voitenok N.N. (2000) Human neutrophil alpha-defensin modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells. *European Cytokine Network* 11:257-266.
- Chamberlain M.C. (2006) Treatment options for glioblastoma. *Neurosurg Focus* 20:E19. DOI: 200402 [pii] 10.3171/foc.2006.20.4.12.
- Chamberlain M.C. (2011) Bevacizumab for the treatment of recurrent glioblastoma. *Clin Med Insights Oncol* 5:117-29. DOI: 10.4137/CMO.S7232 cmo-2011-117 [pii].

- Champoux J.J. (2001) DNA topoisomerases: Structure, function, and mechanism. *Annual Review of Biochemistry* 70:369-413.
- Chang A., Nishiyama A., Peterson J., Prineas J., Trapp B.D. (2000) NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *Journal of Neuroscience* 20:6404-6412.
- Charles N.A., Holland E.C., Gilbertson R., Glass R., Kettenmann H. (2011) The Brain Tumor Microenvironment. *Glia* 59:1169-1180. DOI: Doi 10.1002/Glia.21136.
- Chatterjee N., Stegmüller J., Schatzle P., Karam K., Koroll M., Werner H.B., Nave K.A., Trotter J. (2008) Interaction of synenin-1 and the NG2 proteoglycan in migratory oligodendrocyte precursor cells. *Journal of Biological Chemistry* 283:8310-8317. DOI: DOI 10.1074/jbc.M706074200.
- Chaurand P., Caprioli R.M. (2002) Direct profiling and imaging of peptides and proteins from mammalian cells and tissue sections by mass spectrometry. *Electrophoresis* 23:3125-3135.
- Chaurand P., Stoeckli M., Caprioli R.M. (1999) Direct profiling of proteins in biological tissue sections by MALDI mass spectrometry. *Analytical Chemistry* 71:5263-5270.
- Chavakis T., Cines D.B., Rhee J.S., Liang O.D., Schubert U., Hammes H.P., Higazi A.A., Nawroth P.P., Preissner K.T., Bdeir K. (2004) Regulation of neovascularization by human neutrophil peptides (alpha-defensins): a link between inflammation and angiogenesis. *Faseb Journal* 18:1306-+. DOI: DOI 10.1096/fj.03-1009fje.
- Chekenya M., Hjelstuen M., Enger P.O., Thorsen F., Jacob A.L., Probst B., Haraldseth O., Pilkington G., Butt A., Levine J.M., Bjerkvig R. (2002a) NG2 proteoglycan promotes angiogenesis-dependent tumor growth in CNS by sequestering angiostatin. *FASEB J* 16:586-8.
- Chekenya M., Enger P.O., Thorsen F., Tysnes B.B., Al-Sarraj S., Read T.A., Furmanek T., Mahesparan R., Levine J.M., Butt A.M., Pilkington G.J., Bjerkvig R. (2002b) The glial precursor proteoglycan, NG2, is expressed on tumour neovasculature by vascular pericytes in human malignant brain tumours. *Neuropathology and Applied Neurobiology* 28:367-380.
- Chekenya M., Krakstad C., Svendsen A., Netland I.A., Staalesen V., Tysnes B.B., Selheim F., Wang J., Sakariassen P.O., Sandal T., Lonning P.E., Flatmark T., Enger P.O., Bjerkvig R., Sioud M., Stallcup W.B. (2008) The progenitor cell marker NG2/MPG promotes chemoresistance by activation of integrin-dependent PI3K/Akt signaling. *Oncogene* 27:5182-5194.
- Chen L., Feng P.M., Li S.F., Long D., Cheng J.Q., Lu Y.R., Zhou D. (2009a) Effect of Hypoxia-inducible Factor-1 alpha Silencing on the Sensitivity of Human Brain Glioma Cells to Doxorubicin and Etoposide. *Neurochemical Research* 34:984-990. DOI: 10.1007/s11064-008-9864-9.

- Chen R., Pan S., Brentnall T.A., Aebersold R. (2005) Proteomic profiling of pancreatic cancer for biomarker discovery. *Molecular & Cellular Proteomics* 4:523-533. DOI: DOI 10.1074/mcp.R500004-MCP200.
- Chen Y.D., Zheng S., Yu J.K., Hu X. (2004) Artificial neural networks analysis of surface-enhanced laser desorption/ionization mass spectra of serum protein pattern distinguishes colorectal cancer from healthy population. *Clinical Cancer Research* 10:8380-8385.
- Chen Y.S., Yan W.X., Geczy C.L., Brown M.A., Thomas R. (2009b) Serum levels of soluble receptor for advanced glycation end products and of S100 proteins are associated with inflammatory, autoantibody, and classical risk markers of joint and vascular damage in rheumatoid arthritis. *Arthritis Research & Therapy* 11. DOI: Artn R39 Doi 10.1186/Ar2645.
- Cheng J.X., Liu B.L., Zhang X. (2009) How powerful is CD133 as a cancer stem cell marker in brain tumors? *Cancer Treatment Reviews* 35:403-408. DOI: DOI 10.1016/j.ctrv.2009.03.002.
- Chin L., Meyerson M., Aldape K., Bigner D., Mikkelsen T., VandenBerg S., Kahn A., Penny R., Ferguson M.L., Gerhard D.S., Getz G., Brennan C., Taylor B.S., Winckler W., Park P., Ladanyi M., Hoadley K.A., Verhaak R.G.W., Hayes D.N., Spellman P.T., Absher D., Weir B.A., Ding L., Wheeler D., Lawrence M.S., Cibulskis K., Mardis E., Zhang J.H., Wilson R.K., Donehower L., Wheeler D.A., Purdom E., Wallis J., Laird P.W., Herman J.G., Schuebel K.E., Weisenberger D.J., Baylin S.B., Schultz N., Yao J., Wiedemeyer R., Weinstein J., Sander C., Gibbs R.A., Gray J., Kucherlapati R., Lander E.S., Myers R.M., Perou C.M., McLendon R., Friedman A., Van Meir E.G., Brat D.J., Mastrogiannis G.M., Olson J.J., Lehman N., Yung W.K.A., Bogler O., Berger M., Prados M., Muzny D., Morgan M., Scherer S., Sabo A., Nazareth L., Lewis L., Hall O., Zhu Y.M., Ren Y.R., Alvi O., Yao J.Q., Hawes A., Jhangiani S., Fowler G., San Lucas A., Kovar C., Cree A., Dinh H., Santibanez J., Joshi V., Gonzalez-Garay M.L., Miller C.A., Milosavljevic A., Sougnez C., Fennell T., Mahan S., Wilkinson J., Ziaugra L., Onofrio R., Bloom T., Nicol R., Ardlie K., Baldwin J., Gabriel S., Fulton R.S., McLellan M.D., Larson D.E., Shi X.Q., Abbott R., Fulton L., *et al.* (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455:1061-1068. DOI: Doi 10.1038/Nature07385.
- Chintala S.K., Gokaslan Z.L., Go Y., Sawaya R., Nicolson G.L., Rao J.S. (1996) Role of extracellular matrix proteins in regulation of human glioma cell invasion in vitro. *Clinical & Experimental Metastasis* 14:358-366.
- Choe L., D'Ascenzo M., Relkin N.R., Pappin D., Ross P., Williamson B., Guertin S., Pribil P., Lee K.H. (2007) 8-Plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. *Proteomics* 7:3651-3660.
- Choe L.H., Lee K.H. (2003) Quantitative and qualitative measure of intralaboratory two-dimensional protein gel reproducibility and the effects of sample preparation, sample load, and image analysis. *Electrophoresis* 24:3500-3507. DOI: DOI 10.1002/elps.200305614.

- Ciordia S., de Los Rios V., Albar J.P. (2006) Contributions of advanced proteomics technologies to cancer diagnosis. *Clin Transl Oncol* 8:566-80. DOI: 950 [pii].
- Clarke C.H., Yip C., Badgwell D., Fung E.T., Coombes K.R., Zhang Z., Lu K.H., Bast R.C., Jr. (2011) Proteomic biomarkers apolipoprotein A1, truncated transthyretin and connective tissue activating protein III enhance the sensitivity of CA125 for detecting early stage epithelial ovarian cancer. *Gynecol Oncol* 122:548-53. DOI: S0090-8258(11)00440-9 [pii] 10.1016/j.ygyno.2011.06.002.
- Clarke J., Butowski N., Chang S. (2010) Recent Advances in Therapy for Glioblastoma. *Archives of Neurology* 67:279-283.
- Cloughesy T. (2011) The Impact of Recent Data on the Optimization of Standards of Care in Newly Diagnosed Glioblastoma. *Seminars in Oncology* 38:S11-S20. DOI: DOI 10.1053/j.seminoncol.2011.09.007.
- Colantonio D.A., Chan D.W. (2005) The clinical application of proteomics. *Clinica Chimica Acta* 357:151-158. DOI: DOI 10.1016/j.cccn.2005.03.020.
- Coleman A.M., Rendon B.E., Zhao M., Qian M.W., Bucala R., Xin D., Mitchell R.A. (2008) Cooperative regulation of non-small cell lung carcinoma angiogenic potential by macrophage migration inhibitory factor and its homolog, D-dopachrome tautomerase. *Journal of Immunology* 181:2330-2337.
- Collins V.P. (2004) Brain tumours: classification and genes. *Journal of Neurology, Neurosurgery & Psychiatry* 75 Suppl 2:ii2-11.
- Colnaghi R., Carpenter G., Volker M., O'Driscoll M. (2011) The consequences of structural genomic alterations in humans: Genomic Disorders, genomic instability and cancer. *Semin Cell Dev Biol*. DOI: S1084-9521(11)00096-6 [pii] 10.1016/j.semcdb.2011.07.010.
- Combaret V., Bergeron C., Brejon S., Iacono I., Perol D., Negrier S., Puisieux A. (2005) Protein chip array profiling analysis of sera from neuroblastoma patients. *Cancer Letters* 228:91-96. DOI: DOI 10.1016/j.canlet.2004.12.053.
- Conroy H., Mawhinney L., Donnelly S.C. (2010) Inflammation and cancer: macrophage migration inhibitory factor (MIF)-the potential missing link. *Qjm-an International Journal of Medicine* 103:831-836. DOI: Doi 10.1093/Qjmed/Hcq148.
- Coombes K.R., Fritsche H.A., Clarke C., Chen J.N., Baggerly K.A., Morris J.S., Xiao L.C., Hung M.C., Kuerer H.M. (2003) Quality control and peak finding for proteomics data collected from nipple aspirate fluid by surface-enhanced laser desorption and ionization. *Clinical Chemistry* 49:1615-1623.
- Cortesi L., Rossi E., Della Casa L., Barchetti A., Nicoli A., Piana S., Abrate M., La Sala G.B., Federico M., Iannone A. (2011) Protein expression patterns associated with advanced stage ovarian cancer. *Electrophoresis* 32:1992-2003. DOI: DOI 10.1002/elps.201000654.

- Covello K.L., Kehler J., Yu H.W., Gordan J.D., Arsham A.M., Hu C.J., Labosky P.A., Simon M.C., Keith B. (2006) HIF-2 alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes & Development* 20:557-570. DOI: Doi 10.1101/Gad.1399906.
- Cui D.M., Xu Q.W., Wang K., Che X.M. (2010) Gli1 is a potential target for alleviating multidrug resistance of gliomas. *Journal of the Neurological Sciences* 288:156-166. DOI: 10.1016/j.jns.2009.09.006.
- Cummings L.C., Cooper G.S. (2011) Colorectal Cancer Screening: Update for 2011. *Seminars in Oncology* 38:483-489. DOI: DOI 10.1053/j.seminoncol.2011.05.002.
- Curran W.J., Jr., Scott C.B., Horton J., Nelson J.S., Weinstein A.S., Fischbach A.J., Chang C.H., Rotman M., Asbell S.O., Krisch R.E., et al. (1993) Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. *J Natl Cancer Inst* 85:704-10.
- D'Abaco G.M., Kaye A.H. (2007) Integrins: Molecular determinants of glioma invasion. *Journal of Clinical Neuroscience* 14:1041-1048. DOI: DOI 10.1016/j.jocn.2007.06.019.
- Dang L., White D.W., Gross S., Bennett B.D., Bittinger M.A., Driggers E.M., Fantin V.R., Jang H.G., Jin S., Keenan M.C., Marks K.M., Prins R.M., Ward P.S., Yen K.E., Liao L.M., Rabinowitz J.D., Cantley L.C., Thompson C.B., Heiden M.G.V., Su S.M. (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 462:739-U52. DOI: Doi 10.1038/Nature08617.
- de Martel C., Franceschi S. (2009) Infections and cancer: Established associations and new hypotheses. *Critical Reviews in Oncology Hematology* 70:183-194. DOI: DOI 10.1016/j.critrevonc.2008.07.021.
- De S., Razorenova O., McCabe N.P., O'Toole T., Qin J., Byzova T.V. (2005) VEGF-integrin interplay controls tumor growth and vascularization. *Proc Natl Acad Sci U S A* 102:7589-94. DOI: 0502935102 [pii] 10.1073/pnas.0502935102.
- de Vries N.A., Beijnen J.H., Boogerd W., van Tellingen O. (2006) Blood-brain barrier and chemotherapeutic treatment of brain tumors. *Expert Rev Neurother* 6:1199-209. DOI: 10.1586/14737175.6.8.1199.
- de Wijkerslooth T.R., Bossuyt P.M., Dekker E. (2011) Strategies in screening for colon carcinoma. *Netherlands Journal of Medicine* 69:112-119.
- Decleves X., Fajac A., Lehmann-Che J., Tardy M., Mercier C., Hurbain I., Laplanche J.L., Bernaudin J.F., Scherrmann J.M. (2002) Molecular and functional MDR1-PGP and MRPs expression in human glioblastoma multiforme cell lines. *International Journal of Cancer* 98:173-180. DOI: Doi 10.1002/Ijc.10135.
- Deininger M.H., Pater S., Strik H., Meyermann R. (2001) Macrophage/microglial cell subpopulations in glioblastoma multiforme relapses are differentially altered by radiochemotherapy. *Journal of Neuro-Oncology* 55:141-147.

- Denny B.J., Wheelhouse R.T., Stevens M.F.G., Tsang L.L.H., Slack J.A. (1994) Nmr and Molecular Modeling Investigation of the Mechanism of Activation of the Antitumor Drug Temozolomide and Its Interaction with DNA. *Biochemistry* 33:9045-9051.
- Desai S.D., Liu L.F., Vazquez-Abad D., D'Arpa P. (1997) Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J Biol Chem* 272:24159-64.
- Desgrosellier J.S., Cheresh D.A. (2010) Integrins in cancer: biological implications and therapeutic opportunities. *Nature Reviews Cancer*. 10:9-22.
- Dessein, A.-F., L. Stechly, N. Jonckheere, P. Dumont, D. Monte, E. Leteurtre, S. Truant, F.-R. Pruvot, M. Figeac, M. Hebbar, C.-H. Lecellier, T. Lesuffleur, R. Dessein, G. Grard, M.-J. Dejonghe, Y. de Launoit, Y. Furuichi, G. Prevost, N. Porchet, C. Gespach, and G. Huet. Autocrine induction of invasive and metastatic phenotypes by the MIF-CXCR4 axis in drug-resistant human colon cancer cells. *Cancer Research* 70:4644-54.
- Deweese J.E., Burgin A.B., Osheroff N., Deweese J.E., Burgin A.B., Osheroff N. (2008) Human topoisomerase II α uses a two-metal-ion mechanism for DNA cleavage. *Nucleic Acids Research* 36:4883-93
- Dove A. (1999) Proteomics: translating genomics into products? *Nature Biotechnology* 17:233-6. DOI: 10.1038/6972.
- Drablos F., Feyzi E., Aas P.A., Vaagbo C.B., Kavli B., Bratlie M.S., Pena-Diaz J., Otterlei M., Slupphaug G., Krokan H.E. (2004) Alkylation damage in DNA and RNA - repair mechanisms and medical significance. *DNA Repair* 3:1389-1407. DOI: DOI 10.1016/j.dnarep.2004.05.004.
- Ducray F., Criniere E., Idbaih A., Mokhtari K., Marie Y., Paris S., Navarro S., Laigle-Donadey F., Dehais C., Thillet J., Hoang-Xuan K., Delattre J.Y., Sanson M. (2009) alpha-Internexin expression identifies 1p19q codeleted gliomas. *Neurology* 72:156-161.
- Ducray F., Idbaih A., de Reynies A., Bieche I., Thillet J., Mokhtari K., Lair S., Marie Y., Paris S., Vidaud M., Hoang-Xuan K., Delattre O., Delattre J.Y., Sanson M. (2008) Anaplastic oligodendrogliomas with 1p19q codeletion have a proneural gene expression profile. *Molecular Cancer* 7. DOI: Artn 41 Doi 10.1186/1476-4598-7-41.
- Duffy M.J., Evoy D., McDermott E.W. (2010) CA 15-3: uses and limitation as a biomarker for breast cancer. *Clin Chim Acta* 411:1869-74. DOI: S0009-8981(10)00558-9 [pii] 10.1016/j.cca.2010.08.039.
- Duffy M.J., Bonfrer J.M., Kulpa J., Rustin G.J.S., Soletormos G., Torre G.C., Tuxen M.K., Zwirner M. (2005) CA125 in ovarian cancer: European Group on Tumor Markers guidelines for clinical use. *International Journal of Gynecological Cancer* 15:679-691.
- Dunn I.F., Heese O., Black P.M. (2000) Growth factors in glioma angiogenesis: FGFs, PDGF, EGF, and TGFs. *Journal of Neuro-Oncology* 50:121-137.
- Economopoulou M., Bdeir K., Cines D.B., Fogt F., Bdeir Y., Lubkowski J., Lu W.Y., Preissner K.T., Hammes H.P., Chavakis T. (2005) Inhibition of pathologic retinal

neovascularization by alpha-defensins. *Blood* 106:3831-3838. DOI: DOI 10.1182/blood-2005-03-0889.

Edgeworth J., Gorman M., Bennett R., Freemont P., Hogg N. (1991) Identification of P8,14 as a Highly Abundant Heterodimeric Calcium-Binding Protein Complex of Myeloid Cells. *Journal of Biological Chemistry* 266:7706-7713.

Ehmann M., Felix K., Hartmann D., Schnolzer M., Nees M., Vorderwulbecke S., Bogumil R., Buchler M.W., Friess H. (2007) Identification of potential markers for the detection of pancreatic cancer through comparative serum protein expression profiling. *Pancreas* 34:205-214.

Eisenmann K.M., McCarthy J.B., Simpson M.A., Keely P.J., Guan J.L., Tachibana K., Lim L., Manser E., Furcht L.T., Iida J. (1999) Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130(cas). *Nature Cell Biology* 1:507-513.

Endicott J.A., Ling V. (1989) The Biochemistry of P-Glycoprotein-Mediated Multidrug Resistance. *Annual Review of Biochemistry* 58:137-171.

Engwegen J.Y., Mehra N., Haanen J.B., Schellens J.H., Voest E.E., Beijnen J.H., Engwegen J.Y.M.N., Mehra N., Haanen J.B.A.G., Schellens J.H.M., Voest E.E., Beijnen J.H. (2009) Identification of two new serum protein profiles for renal cell carcinoma. *Oncology Reports* 22:401-8.

Engwegen J.Y.M.N., Helgason H.H., Cats A., Harris N., Bonfrer J.M.G., Schellens J.H.M., Beijnen J.H. (2006) Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. *World Journal of Gastroenterology* 12:1536-1544.

Engwegen J.Y.M.N., Mehra N., Haanen J.B.A.G., Bonfrer J.M.G., Schellen J.H.M., Voest E.E., Beijnen J.H. (2007) Validation of SELDI-TOF MS serum protein profiles for renal cell carcinoma in new populations. *Laboratory Investigation* 87:161-172. DOI: DOI 10.1038/labinvest.3700503.

Escher N., Spies-Weisshart B., Kaatz M., Melle C., Bleul A., Driesch D., Wollina U., Von Eggeling F. (2006) Identification of HNP3 as a tumour marker in CD4+ and CD4- lymphocytes of patients with cutaneous T-cell lymphoma. *European Journal of Cancer* 42:249-255. DOI: DOI 10.1016/j.ejca.2005.07.033.

Esteller M., Hamilton S.R., Burger P.C., Baylin S.B., Herman J.G. (1999) Inactivation of the DNA repair gene O-6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Research* 59:793-797.

Esteller M., Garcia-Foncillas J., Andion E., Goodman S.N., Hidalgo O.F., Vanaclocha V., Baylin S.B., Herman J.G. (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *New England Journal of Medicine* 343:1350-1354.

- Everhard S., Tost J., El Abdalaoui H., Criniere E., Busato F., Marie Y., Gut I.G., Sanson M., Mokhtari K., Laigle-Donadey F., Hoang-Xuan K., Delattre J.Y., Thillet J. (2009) Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro-Oncology* 11:348-356. DOI: Doi 10.1215/15228517-2009-001.
- Fan J.R., Peng A.L., Chen H.C., Lo S.C., Huang T.H., Li T.K., Fan J.-R., Peng A.-L., Chen H.-C., Lo S.-C., Huang T.-H., Li T.-K. (2008) Cellular processing pathways contribute to the activation of etoposide-induced DNA damage responses. *DNA Repair* 7:452-63.
- Fang X.X., Burg M.A., Barritt D., Dahlin-Huppe K., Nishiyama A., Stallcup W.B. (1999) Cytoskeletal reorganization induced by engagement of the NG2 proteoglycan leads to cell spreading and migration. *Molecular Biology of the Cell* 10:3373-3387.
- Felix K., Fakelman F., Hartmann D., Giese N.A., Gaida M.M., Schnolzer M., Flad T., Buchler M.W., Werner J. (2011) Identification of serum proteins involved in pancreatic cancer cachexia. *Life Sciences* 88:218-225. DOI: DOI 10.1016/j.lfs.2010.11.011.
- Ferguson S.D. (2011) Malignant Gliomas: Diagnosis and Treatment. *Dm Disease-a-Month* 57:558-569. DOI: DOI 10.1016/j.disamonth.2011.08.020.
- Ferrer-Luna R., Mata M., Nunez L., Calvar J., Dasi F., Arias E., Piquer J., Cerda-Nicolas M., Taratuto A.L., Sevlever G., Celda B., Martinetto H. (2009) Loss of heterozygosity at 1p-19q induces a global change in oligodendroglial tumor gene expression. *Journal of Neuro-Oncology* 95:343-354. DOI: DOI 10.1007/s11060-009-9944-y.
- Filippini G., Falcone C., Boiardi A., Broggi G., Bruzzzone M.G., Caldiroli D., Farina R., Farinotti M., Fariselli L., Finocchiaro G., Giombini S., Polio B., Savoiaro M., Solero C.L., Valsecchi M.G., Ist B.C.R.F.I. (2008) Prognostic factors for survival in 676 consecutive patients with newly diagnosed primary glioblastoma. *Neuro-Oncology* 10:79-87. DOI: Doi 10.1215/15228517-2007-038.
- Fine H.A., Dear K.B.G., Loeffler J.S., Black P.M., Canellos G.P. (1993) Metaanalysis of Radiation-Therapy with and without Adjuvant Chemotherapy for Malignant Gliomas in Adults. *Cancer* 71:2585-2597.
- Foell D., Roth J. (2004) Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis and Rheumatism* 50:3762-3771. DOI: Doi 10.1002/Art.20631.
- Fojo T., Bates S. (2003) Strategies for reversing drug resistance. *Oncogene* 22:7512-7523. DOI: DOI 10.1038/sj.onc.1206951.
- Forshed J., Pernemalm M., Tan C.S., Lindberg M., Kanter L., Pawitan Y., Lewensohn R., Stenke L., Lehtio J. (2008) Proteomic data analysis workflow for discovery of candidate biomarker peaks predictive of clinical outcome for patients with acute myeloid leukemia. *Journal of Proteome Research* 7:2332-2341. DOI: Doi 10.1021/P00482e.
- Fortin D., Gendron C., Boudrias M., Garant M.P. (2007) Enhanced chemotherapy delivery by intraarterial infusion and blood-brain barrier disruption in the treatment of cerebral metastasis. *Cancer* 109:751-760. DOI: Doi 10.1002/Cncr.22450.

- Fortune J.M., Osherooff N. (2000) Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice. *Progress in Nucleic Acid Research and Molecular Biology*, Vol 64 64:221-253.
- Fossati G., Ricevuti G., Edwards S.W., Walker C., Dalton A., Rossi M.L. (1999) Neutrophil infiltration into human gliomas. *Acta Neuropathologica* 98:349-354.
- Franceschi E., Cavallo G., Scopece L., Paioli A., Pession A., Magrini E., Conforti R., Palmerini E., Bartolini S., Rimondini S., Esposti R.D., Crino L., Esposti R.D. (2004) Phase II trial of carboplatin and etoposide for patients with recurrent high-grade glioma. *British Journal of Cancer* 91:1038-44.
- Freedman V.H., Shin S. (1974) Cellular Tumorigenicity in Nude Mice - Correlation with Cell-Growth in Semisolid Medium. *Cell* 3:355-359.
- Friedman H.S., McLendon R.E., Kerby T., Dugan M., Bigner S.H., Henry A.J., Ashley D.M., Krischer J., Lovell S., Rasheed K., Marchev F., Seman A.J., Cokgor I., Rich J., Stewart E., Colvin O.M., Provenzale J.M., Bigner D.D., Haglund M.M., Friedman A.H., Modrich P.L. (1998) DNA mismatch repair and O-6-alkylguanine-DNA alkyltransferase analysis and response to temodal in newly diagnosed malignant glioma. *Journal of Clinical Oncology* 16:3851-3857.
- Frosch M., Roth J. (2008) New insights in systemic juvenile idiopathic arthritis from pathophysiology to treatment. *Rheumatology* 47:121-125. DOI: DOI 10.1093/rheumatology/kem271.
- Fukushi J., Inatani M., Yamaguchi Y., Stallcup W.B. (2003) Expression of NG2 proteoglycan during endochondral and intramembranous ossification. *Developmental Dynamics* 228:143-148. DOI: Doi 10.1002/Dvdy.10359.
- Fukushi J., Makagiansar I.T., Stallcup W.B., Fukushi J.-i., Makagiansar I.T., Stallcup W.B. (2004) NG2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. *Molecular Biology of the Cell* 15:3580-90.
- Fulton D., Urtasun R., Forsyth P. (1996a) Phase II study of prolonged oral therapy with etoposide (VP16) for patients with recurrent malignant glioma. *J Neurooncol* 27:149-55.
- Fulton D., Urtasun R., Forsyth P. (1996b) Phase II study of prolonged oral therapy with etoposide (VP16) for patients with recurrent malignant glioma. *Journal of Neuro-Oncology* 27:149-55.
- Furnari F.B., Fenton T., Bachoo R.M., Mukasa A., Stommel J.M., Stegh A., Hahn W.C., Ligon K.L., Louis D.N., Brennan C., Chin L., DePinho R.A., Cavenee W.K., Furnari F.B., Fenton T., Bachoo R.M., Mukasa A., Stommel J.M., Stegh A., Hahn W.C., Ligon K.L., Louis D.N., Brennan C., Chin L., DePinho R.A., Cavenee W.K. (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes & Development* 21:2683-710.

- Ganz T., Selsted M.E., Szklarek D., Harwig S.S.L., Daher K., Bainton D.F., Lehrer R.I. (1985) Defensins - Natural Peptide Antibiotics of Human-Neutrophils. *Journal of Clinical Investigation* 76:1427-1435.
- Gast M.C., van Dulken E.J., van Loenen T.K., Kingma-Vegter F., Westerga J., Flohil C.C., Knol J.C., Jimenez C.R., van Gils C.H., Wessels L.F., Schellens J.H., Beijnen J.H. (2009a) Detection of breast cancer by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry tissue and serum protein profiling. *Int J Biol Markers* 24:130-41. DOI: D2599077-0BCB-40D8-BA4D-C5AD52F8C7F2 [pii].
- Gast M.C.W., Schellens J.H.M., Beijnen J.H. (2009b) Clinical proteomics in breast cancer: a review. *Breast Cancer Research and Treatment* 116:17-29. DOI: DOI 10.1007/s10549-008-0263-3.
- Gast M.C.W., Van Gils C.H., Wessels L.F.A., Harris N., Bonfrer J.M.G., Rutgers E.J.T., Schellens J.H.M., Beijnen J.H. (2009c) Serum protein profiling for diagnosis of breast cancer using SELDI-TOF MS. *Oncology Reports* 22:205-213. DOI: Doi 10.3892/or_00000426.
- Gatlin-Bunai C.L., Cazares L.H., Cooke W.E., Semmes O.J., Malyarenko D.I. (2007) Optimization of MALDI-TOF MS detection for enhanced sensitivity of affinity-captured proteins spanning a 100 kDa mass range. *Journal of Proteome Research* 6:4517-4524. DOI: Doi 10.1021/Pr0703526.
- Gebhardt C., Breitenbach U., Tuckermann J.P., Dittrich B.T., Richter K.H., Angel P. (2002) Calgranulins S100A8 and S100A9 are negatively regulated by glucocorticoids in a c Fos-dependent manner and overexpressed throughout skin carcinogenesis. *Oncogene* 21:4266-4276. DOI: DOI 10.1038/sj.onc.1205521.
- Gebhardt C., Riehl A., Durchdewald M., Nemeth J., Furstenberger G., Muller-Decker K., Enk A., Arnold B., Bierhaus A., Nawroth P.P., Hess J., Angel P. (2008) RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med* 205:275-85. DOI: jem.20070679 [pii] 10.1084/jem.20070679.
- Getie M., Schmelzer C.E.H., Weiss A.S., Neubert R.H.H. (2005) Complementary mass spectrometric techniques to achieve complete sequence coverage of recombinant human tropoelastin. *Rapid Communications in Mass Spectrometry* 19:2989-2993. DOI: Doi 10.1002/Rcm.2164.
- Giard D.J., Aaronson S.A., Todaro G.J., Arnstein P., Kersey J.H., Dosik H., Parks W.P. (1973) In-Vitro Cultivation of Human Tumors - Establishment of Cell Lines Derived from a Series of Solid Tumors. *Journal of the National Cancer Institute* 51:1417-1423.
- Gilbert M.R., Kuhn J., Lamborn K.R., Lieberman F., Wen P.Y., Mehta M., Cloughesy T., Lassman A.B., DeAngelis L.M., Chang S., Prados M. (2012) Cilengitide in patients with recurrent glioblastoma: the results of NABTC 03-02, a phase II trial with measures of treatment delivery. *Journal of Neuro-Oncology* 106:147-153. DOI: DOI 10.1007/s11060-011-0650-1.
- Gillet J.P., Gottesman M.M. (2010) Mechanisms of multidrug resistance in cancer. *Methods Mol Biol* 596:47-76. DOI: 10.1007/978-1-60761-416-6_4.

- Gingras M.C., Roussel E., Bruner J.M., Branch C.D., Moser R.P. (1995) Comparison of Cell-Adhesion Molecule Expression between Glioblastoma-Multiforme and Autologous Normal Brain-Tissue. *Journal of Neuroimmunology* 57:143-153.
- Gonnet F., Lemaitre G., Waksman G., Tortajada J. (2003) MALDI/MS peptide mass fingerprinting for proteome analysis: identification of hydrophobic proteins attached to eucaryote keratinocyte cytoplasmic membrane using different matrices in concert. *Proteome Sci* 1:2.
- Goretzki L., Lombardo C.R., Stallcup W.B. (2000) Binding of the NG2 proteoglycan to kringle domains modulates the functional properties of angiostatin and plasmin(ogen). *Journal of Biological Chemistry* 275:28625-28633.
- Goretzki L., Burg M.A., Grako K.A., Stallcup W.B. (1999) High-affinity binding of basic fibroblast growth factor and platelet-derived growth factor-AA to the core protein of the NG2 proteoglycan. *Journal of Biological Chemistry* 274:16831-16837.
- Gottesman M.M., Pastan I. (1993) Biochemistry of Multidrug-Resistance Mediated by the Multidrug Transporter. *Annual Review of Biochemistry* 62:385-427.
- Goyette J., Geczy C.L. (2009) Inflammation-associated S100 proteins: new mechanisms that regulate function. *Amino Acids* 37:77-77.
- Grako K.A., Stallcup W.B. (1995) Participation of the NG2 proteoglycan in rat aortic smooth muscle cell responses to platelet-derived growth factor. *Exp Cell Res* 221:231-40. DOI: S0014-4827(85)71371-7 [pii] 10.1006/excr.1995.1371.
- Griffin C.A., Burger P., Morsberger L., Yonescu R., Swierczynski S., Weingart J.D., Murphy K.M. (2006) Identification of der(1;19)(q10;p10) in five oligodendrogliomas suggests mechanism of concurrent 1p and 19q loss. *Journal of Neuropathology and Experimental Neurology* 65:988-994.
- Grigat J., Soruri A., Forssmann U., Riggert J., Zwirner J. (2007) Chemoattraction of macrophages, T lymphocytes, and mast cells is evolutionarily conserved within the human alpha-defensin family. *Journal of Immunology* 179:3958-3965.
- Grossman S.A., Osman M., Hruban R., Piantadosi S. (1999) Central nervous system cancers in first-degree relatives and spouses. *Cancer Investigation* 17:299-308.
- Grus F.H., Podust V.N., Bruns K., Lackner K., Fu S.Y., Dalmaso E.A., Wirthlin A., Pfeiffer N. (2005) SELDI-TOF-MS ProteinChip Array profiling of tears from patients with dry eye. *Investigative Ophthalmology & Visual Science* 46:863-876. DOI: Doi 10.1167/Iovs.04-0448.
- Gudkov A.V., Zelnick C.R., Kazarov A.R., Thimmapaya R., Suttle D.P., Beck W.T., Roninson I.B. (1993a) Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA. *Proc Natl Acad Sci U S A* 90:3231-5.
- Gudkov A.V., Zelnick C.R., Kazarov A.R., Thimmapaya R., Suttle D.P., Beck W.T., Roninson I.B. (1993b) Isolation of genetic suppressor elements, inducing resistance to

- topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA. *Proceedings of the National Academy of Sciences of the United States of America* 90:3231-5.
- Guo Z., Zhang Q.C., Zou H.F., Guo B.C., Ni J.Y. (2002) A method for the analysis of low-mass molecules by MALDI-TOF mass spectrometry. *Analytical Chemistry* 74:1637-1641. DOI: Doi 10.1021/Ac010979m.
- Gupta T., Dinshaw K. (2005) Modified optimal fractionation for poor prognosis malignant gliomas: An elusive search. *Acta Oncologica* 44:105-113.
- Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H., Aebersold R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnology* 17:994-999.
- Han K.Q., Huang G., Gao C.F., Wang X.L., Ma B., Sun L.Q., Wei Z.J., Han K.-Q., Huang G., Gao C.-F., Wang X.-L., Ma B., Sun L.-Q., Wei Z.-J. (2008) Identification of lung cancer patients by serum protein profiling using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. *American Journal of Clinical Oncology* 31:133-9.
- Hanash S. (2003) Disease proteomics. *Nature* 422:226-232. DOI: Doi 10.1038/Nature01514.
- Hanley J.A., Mcneil B.J. (1982) The Meaning and Use of the Area under a Receiver Operating Characteristic (Roc) Curve. *Radiology* 143:29-36.
- Hanley J.A., Mcneil B.J. (1983) A Method of Comparing the Areas under Receiver Operating Characteristic Curves Derived from the Same Cases. *Radiology* 148:839-843.
- Hansen K.C., Schmitt-Ulms G., Chalkley R.J., Hirsch J., Baldwin M.A., Burlingame A.L. (2003) Mass spectrometric analysis of protein mixtures at low levels using cleavable C-13-isotope-coded affinity tag and multidimensional chromatography. *Molecular & Cellular Proteomics* 2:299-314. DOI: DOI 10.1074/mcp.M#00021-MCP200.
- Harbaugh K.S., Black P.M. (1998) Strategies in the surgical management of malignant gliomas. *Seminars in Surgical Oncology* 14:26-33.
- Haylock-Jacobs S., Keough M.B., Lau L., Yong V.W. (2011) Chondroitin sulphate proteoglycans: Extracellular matrix proteins that regulate immunity of the central nervous system. *Autoimmunity Reviews* 10:766-772. DOI: DOI 10.1016/j.autrev.2011.05.019.
- He Q.Y., Chiu J.F. (2003) Proteomics in biomarker discovery and drug development. *Journal of Cellular Biochemistry* 89:868-886. DOI: Doi 10.1002/Jcb.10576.
- Hegi M.E., Liu L., Herman J.G., Stupp R., Wick W., Weller M., Mehta M.P., Gilbert M.R. (2008) Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J Clin Oncol* 26:4189-99. DOI: 26/25/4189 [pii] 10.1200/JCO.2007.11.5964.

- Hegi M.E., Diserens A.C., Godard S., Dietrich P.Y., Regli L., Ostermann S., Otten P., Van Melle G., de Tribolet N., Stupp R. (2004) Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. *Clinical Cancer Research* 10:1871-1874.
- Hegi M.E., Diserens A., Gorlia T., Hamou M., de Tribolet N., Weller M., Kros J.M., Hainfellner J.A., Mason W., Mariani L., Bromberg J.E.C., Hau P., Mirimanoff R.O., Cairncross J.G., Janzer R.C., Stupp R. (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. *New England Journal of Medicine* 352:997-1003.
- Heike Y., Hosokawa M., Osumi S., Fujii D., Aogi K., Takigawa N., Ida M., Tajiri H., Eguchi K., Shiwa M., Wakatabe R., Arikuni H., Takaue Y., Takashima S. (2005) Identification of serum proteins related to adverse effects induced by docetaxel infusion from protein expression profiles of serum using SELDI ProteinChip system. *Anticancer Research* 25:1197-1203.
- Heldin C.H. (1992) Structural and Functional-Studies on Platelet-Derived Growth-Factor. *Embo Journal* 11:4251-4259.
- Helgason H.H., Engwegen J.Y.M.N., Zapatka M., Vincent A., Cats A., Boot H., Beijnen J.H., Schellens J.H.M. (2010) Identification of serum proteins as prognostic and predictive markers of colorectal cancer using surface enhanced laser desorption ionization-time of flight mass spectrometry. *Oncology Reports* 24:57-64. DOI: Doi 10.3892/or_00000828.
- Hill C.P., Yee J., Selsted M.E., Eisenberg D. (1991) Crystal-Structure of Defensin Hnp-3, an Amphiphilic Dimer - Mechanisms of Membrane Permeabilization. *Science* 251:1481-1485.
- Hira E., Ono T., Dhar D.K., El-Assal O.N., Hishikawa Y., Yamanoi A., Nagasue N. (2005) Overexpression of macrophage migration inhibitory factor induces angiogenesis and deteriorates prognosis after radical resection for hepatocellular carcinoma. *Cancer* 103:588-598. DOI: Doi 10.1002/Cncr.20818.
- Hiratsuka S., Watanabe A., Sakurai Y., Akashi-Takamura S., Ishibashi S., Miyake K., Shibuya M., Akira S., Aburatani H., Maru Y. (2008) The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nature Cell Biology* 10:1349-U229. DOI: Doi 10.1038/Ncb1794.
- Hobbs S.K., Shi G., Homer R., Harsh G., Atlas S.W., Bednarski M.D. (2003) Magnetic resonance image-guided proteomics of human glioblastoma multiforme. *J Magn Reson Imaging* 18:530-6. DOI: 10.1002/jmri.10395.
- Hollstein M., Sidransky D., Vogelstein B., Harris C.C. (1991) P53 Mutations in Human Cancers. *Science* 253:49-53.
- Holterman D.A., Diaz J.I., Blackmore P.F., Davis J.W., Schellhammer P.F., Corica A., Semmes O.J., Vlahou A. (2006) Overexpression of alpha-defensin is associated with bladder cancer invasiveness. *Urologic Oncology-Seminars and Original Investigations* 24:97-108. DOI: DOI 10.1016/j.urolonc.2005.07.010.

- Honda K., Hayashida Y., Umaki T., Okusaka T., Kosuge T., Kikuchi S., Endo M., Tsuchida A., Aoki T., Itoi T., Moriyasu F., Hirohashi S., Yamada T. (2005) Possible detection of pancreatic cancer by plasma protein profiling. *Cancer Research* 65:10613-10622. DOI: Doi 10.1158/0008-5472.Can-05-1851.
- Hovinga K.E., Stalpers L.J.A., van Bree C., Donker M., Verhoeff J.J.C., Rodermond H.M., Bosch D.A., van Furth W.R. (2005) Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines - a clue to radioresistance? *Journal of Neuro-Oncology* 74:99-103. DOI: DOI 10.1007/s11060-004-4204-7.
- Hu S.P., Harrison C., Xu K., Cornish C.J., Geczy C.L. (1996) Induction of the chemotactic S100 protein, CP-10, in monocyte macrophages by lipopolysaccharide. *Blood* 87:3919-3928.
- Hubert P., Herman L., Maillard C., Caberg J.H., Nikkels A., Pierard G., Foidart J.M., Noel A., Boniver J., Delvenne P. (2007) Defensins induce the recruitment of dendritic cells in cervical human papillomavirus-associated (pre) neoplastic lesions formed in vitro and transplanted in vivo. *Faseb Journal* 21:2765-2775. DOI: DOI 10.1096/fj.06-7646com.
- Hueber W., Robinson W.H. (2006) Proteomic biomarkers for autoimmune disease. *Proteomics* 6:4100-4105. DOI: DOI 10.1002/pmic.200600017.
- Huse J.T., Holland E.C. (2010) Targeting brain cancer: advances in the molecular pathology of malignant glioma and medulloblastoma. *Nature Reviews Cancer* 10:319-331.
- Hutchens T.W., Yip T.T. (1992) Synthetic Metal-Binding Protein Surface Domains for Metal Ion-Dependent Interaction Chromatography .2. Immobilization of Synthetic Metal-Binding Peptides from Metal-Ion Transport Proteins as Model Bioactive Protein Surface Domains. *Journal of Chromatography* 604:133-141.
- Hutchens T.W., Yip T.T. (1993) New Desorption Strategies for the Mass-Spectrometric Analysis of Macromolecules. *Rapid Communications in Mass Spectrometry* 7:576-580.
- Hutchens T.W., Nelson R.W., Li C.M., Yip T.T. (1992) Synthetic Metal-Binding Protein Surface Domains for Metal Ion-Dependent Interaction Chromatography .1. Analysis of Bound Metal-Ions by Matrix-Assisted Uv-Laser Desorption Time-of-Flight Mass-Spectrometry. *Journal of Chromatography* 604:125-132.
- Ichikawa M., Williams R., Wang L., Vogl T., Srikrishna G. (2011) S100A8/A9 Activate Key Genes and Pathways in Colon Tumor Progression. *Molecular Cancer Research* 9:133-148. DOI: Doi 10.1158/1541-7786.Mcr-10-0394.
- Ichimura K., Vogazianou A.P., Liu L., Pearson D.M., Backlund L.M., Plant K., Baird K., Langford C.F., Gregory S.G., Collins V.P. (2008) 1p36 is a preferential target of chromosome 1 deletions in astrocytic tumours and homozygously deleted in a subset of glioblastomas. *Oncogene* 27:2097-2108. DOI: DOI 10.1038/sj.onc.1210848.
- Idbaih A., Marie Y., Pierron G., Brennetot C., Khe H.X., Kujas M., Mokhtari K., Sanson M., Lejeune J., Aurias A., Delattre O., Delattre J.Y. (2005) Two types of chromosome 1p

losses with opposite significance in gliomas. *Annals of Neurology* 58:483-487. DOI: Doi 10.1002/Ana.20607.

Idbaih A., Marie Y., Lucchesi C., Pierron G., Manie E., Raynal V., Mosseri V., Hoang-Xuan K., Kujas M., Brito I., Mokhtari K., Sanson M., Barillot E., Aurias A., Delattre J.Y., Delattre O. (2008) BAC array CGH distinguishes mutually exclusive alterations that define clinicogenetic subtypes of gliomas. *International Journal of Cancer* 122:1778-1786. DOI: Doi 10.1002/Ijc.23270.

Iida J., Meijne A.M.L., Spiro R.C., Roos E., Furcht L.T., Mccarthy J.B. (1995) Spreading and Focal Contact Formation of Human-Melanoma Cells in Response to the Stimulation of Both Melanoma-Associated Proteoglycan (Ng2) and Alpha-4-Beta-1 Integrin. *Cancer Research* 55:2177-2185.

Iida J., Pei D., Kang T., Simpson M.A., Herlyn M., Furcht L.T., McCarthy J.B. (2001) Melanoma chondroitin sulfate proteoglycan regulates matrix metalloproteinase-dependent human melanoma invasion into type I collagen. *Journal of Biological Chemistry* 276:18786-18794.

Imai K., Koshiyama A., Nakata K. (2011) Towards clinical proteomics analysis. *Biomedical Chromatography* 25:59-64. DOI: Doi 10.1002/Bmc.1541.

Ishii N., Maier D., Merlo A., Tada M., Sawamura Y., Diserens A.C., Van Meir E.G. (1999) Frequent co-alterations of TP53, p16/CDKN2A, p14(ARF), PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathology* 9:469-479.

Issaq H.J. (2001) The role of separation science in proteomics research. *Electrophoresis* 22:3629-3638.

Issaq H.J., Veenstra T.D. (2008) Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. *Biotechniques* 44:697-+. DOI: Doi 10.2144/000112823.

Issaq H.J., Veenstra T.D., Conrads T.P., Felschow D. (2002) The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *Biochemical and Biophysical Research Communications* 292:587-592. DOI: DOI 10.1006/bbrc.2002.6678.

Issaq H.J., Conrads T.P., Prieto D.A., Tirumalai R., Veenstra T.D., Issaq H.J., Conrads T.P., Prieto D.A., Tirumalai R., Veenstra T.D. (2003) SELDI-TOF MS for diagnostic proteomics. *Analytical Chemistry* 75:148A-155A.

Iwamoto F.M., Lamborn K.R., Robins H.I., Mehta M.P., Chang S.M., Butowski N.A., Deangelis L.M., Abrey L.E., Zhang W.-T., Prados M.D., Fine H.A. (2010) Phase II trial of pazopanib (GW786034), an oral multi-targeted angiogenesis inhibitor, for adults with recurrent glioblastoma (North American Brain Tumor Consortium Study 06-02). *Neuro-Oncology* 12:855-61.

Jaeckle K.A., Eyre H.J., Townsend J.J., Schulman S., Knudson H.M., Belanich M., Yarosh D.B., Bearman S.I., Giroux D.J., Schold S.C. (1998) Correlation of tumor O-6 methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma

patients treated with bis-chloroethylnitrosourea: A Southwest Oncology Group study. *Journal of Clinical Oncology* 16:3310-3315.

- Jarvinen T.A.H., Tanner M., Rantanen V., Barlund M., Borg A., Grenman S., Isola J. (2000) Amplification and deletion of topoisomerase II alpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. *American Journal of Pathology* 156:839-847.
- Jenkins R.B., Blair H., Ballman K.V., Giannini C., Arusell R.M., Law M., Flynn H., Passe S., Felten S., Brown P.D., Shaw E.G., Buckner J.C. (2006) A t(1;19)(q10;p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. *Cancer Research* 66:9852-9861. DOI: Doi 10.1158/0008-5472.Can-06-1796.
- Jeuken J.W., Cornelissen S.J.B., Vriezen M., Dekkers M.M.G., Errami A., Sijben A., Boots-Sprenger S.H.E., Wesseling P. (2007) MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas. *Laboratory Investigation* 87:1055-1065. DOI: DOI 10.1038/labinvest.3700664.
- Jin S.F., Inoue S., Weaver D.T. (1998) Differential etoposide sensitivity of cells deficient in the Ku and DNA-PKcs components of the DNA-dependent protein kinase. *Carcinogenesis* 19:965-971.
- Jones L.L., Sajed D., Tuszynski M.H. (2003) Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: A balance of permissiveness and inhibition. *Journal of Neuroscience* 23:9276-9288.
- Juillerat-Jeanneret L., Bernasconi C.C., Bricod C., Gros S., Trepey S., Benhattar J., Janzer R.C. (2008) Heterogeneity of human glioblastoma: Glutathione-S-transferase and methylguanine-methyltransferase. *Cancer Investigation* 26:597-609. DOI: Doi 10.1080/07357900802072913.
- Kadoya K., Fukushi J.I., Matsumoto Y., Yamaguchi Y., Stallcup W.B. (2008) NG2 proteoglycan expression in mouse skin: Altered postnatal skin development in the NG2 null mouse. *Journal of Histochemistry & Cytochemistry* 56:295-303. DOI: DOI 10.1369/jhc.7A7349.2007.
- Kaina B., Christmann M., Naumann S., Roos W.P. (2007) MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair* 6:1079-1099. DOI: DOI 10.1016/j.dnarep.2007.03.008.
- Kalinina J., Peng J.M., Ritchie J.C., Van Meir E.G. (2011a) Proteomics of gliomas: Initial biomarker discovery and evolution of technology. *Neuro-Oncology* 13:926-942. DOI: DOI 10.1093/neuonc/nor078.
- Kalinina J., Peng J., Ritchie J.C., Van Meir E.G. (2011b) Proteomics of gliomas: initial biomarker discovery and evolution of technology. *Neuro Oncol* 13:926-42. DOI: nor078 [pii] 10.1093/neuonc/nor078.

- Kanzawa T., Germano I.M., Komata T., Ito H., Kondo Y., Kondo S. (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death and Differentiation* 11:448-457. DOI: DOI 10.1038/sj.cdd.4401359.
- Karas M., Hillenkamp F. (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Analytical Chemistry* 60:2299-301.
- Karas M., Bachmann D., Hillenkamp F. (1985) Influence of the Wavelength in High-Irradiance Ultraviolet-Laser Desorption Mass-Spectrometry of Organic-Molecules. *Analytical Chemistry* 57:2935-2939.
- Karayan-Tapon L., Quillien V., Guilhot J., Wager M., Fromont G., Saikali S., Etcheverry A., Hamlat A., Loussouarn D., Campion L., Campone M., Vallette F.M., Gratas-Rabbia-Re C. (2010) Prognostic value of O(6)-methylguanine-DNA methyltransferase status in glioblastoma patients, assessed by five different methods. *Journal of Neuro-Oncology* 97:311-322. DOI: DOI 10.1007/s11060-009-0031-1.
- Kato S., Han S.Y., Liu W., Otsuka K., Shibata H., Kanamaru R., Ishioka C. (2003) Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. *Proc Natl Acad Sci U S A* 100:8424-9. DOI: 10.1073/pnas.1431692100 1431692100 [pii].
- Kaufmann S.H., Karp J.E., Jones R.J., Miller C.B., Schneider E., Zwelling L.A., Cowan K., Wendel K., Burke P.J. (1994) Topoisomerase-II Levels and Drug-Sensitivity in Adult Acute Myelogenous Leukemia. *Blood* 83:517-530.
- Keles G.E., Lamborn K.R., Chang S.M., Prados M.D., Berger M.S. (2004) Volume of residual disease as a predictor of outcome in adult patients with recurrent supratentorial glioblastomas multiforme who are undergoing chemotherapy. *Journal of Neurosurgery* 100:41-46.
- Kellner U., Heidebrecht H.J., Rudolph P., Biersack H., Buck F., Dakowski T., Wacker H.H., Domanowski M., Seidel A., Westergaard O., Parwaresch R. (1997) Detection of human topoisomerase II alpha in cell lines and tissues: characterization of five novel monoclonal antibodies. *Journal of Histochemistry & Cytochemistry* 45:251-63.
- Khasraw M., McCowatt S.A., Kerestes Z., Buyse M.E., Back M., Wheeler H. (2011) Radiotherapy (RT), temozolomide (TMZ), procarbazine (PCB), and the integrin inhibitor cilengitide in patients with glioblastoma (GBM) without methylation of the MGMT gene promoter (ExCentric) [suppl; abstract TPS133]. *J Clin Oncol* 29.
- Khine A.A., Del Sorbo L., Vaschetto R., Voglis S., Tullis E., Slutsky A.S., Downey G.P., Zhang H.B. (2006) Human neutrophil peptides induce interleukin-8 production through the P2Y(6) signaling pathway. *Blood* 107:2936-2942. DOI: DOI 10.1182/blood-2005-06-2314.
- Khwaja F.W., Nolen J.D., Mendrinios S.E., Lewis M.M., Olson J.J., Pohl J., Van Meir E.G., Ritchie J.C., Brat D.J., Khwaja F.W., Nolen J.D., Mendrinios S.E., Lewis M.M., Olson J.J., Pohl J., Van Meir E.G., Ritchie J.C., Brat D.J. (2006) Proteomic analysis of

cerebrospinal fluid discriminates malignant and nonmalignant disease of the central nervous system and identifies specific protein markers. *Proteomics* 6:6277-87.

Kim H.J., Kang H.J., Lee H., Lee S.T., Yu M.H., Kim H., Lee C. (2009) Identification of S100A8 and S100A9 as Serological Markers for Colorectal Cancer. *Journal of Proteome Research* 8:1368-1379. DOI: Doi 10.1021/Pr8007573.

Kim J.H., Zheng L.T., Lee W.H., Suk K. (2011a) Pro-apoptotic role of integrin beta 3 in glioma cells. *Journal of Neurochemistry* 117:494-503. DOI: DOI 10.1111/j.1471-4159.2011.07219.x.

Kim W.J., Kim S.K., Jeong P., Yun S.J., Cho I.C., Kim I.Y., Moon S.K., Um H.D., Choi Y.H. (2011b) A Four-Gene Signature Predicts Disease Progression in Muscle Invasive Bladder Cancer. *Molecular Medicine* 17:478-485. DOI: DOI 10.2119/molmed.2010.00274.

Kiya K., Uozumi T., Ogasawara H., Sugiyama K., Hotta T., Mikami T., Kurisu K. (1992) Penetration of etoposide into human malignant brain tumors after intravenous and oral administration. *Cancer Chemotherapy & Pharmacology* 29:339-42.

Kleihues P., Ohgaki H. (1999) Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro-Oncology* 1:44-51.

Kodadek T. (2001) Protein microarrays: prospects and problems. *Chemistry & Biology* 8:105-115.

Koopmann J., Zhang Z., White N., Rosenzweig J., Fedarko N., Jagannath S., Canto M.I., Yeo C.J., Chan D.W., Goggins M. (2004) Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization mass spectrometry. *Clinical Cancer Research* 10:860-868.

Koskimaa H.M., Kurvinen K., Costa S., Syrjanen K., Syrjanen S. (2010) Molecular Markers Implicating Early Malignant Events in Cervical Carcinogenesis. *Cancer Epidemiology Biomarkers & Prevention* 19:2003-2012. DOI: Doi 10.1158/1055-9965.Epi-09-0781.

Kostron H., Bauer R. (2011) Management of recurrent malignant glioma--neurosurgical strategies. *Wien Med Wochenschr* 161:20-1. DOI: 10.1007/s10354-010-0861-7.

Kozak K.R., Su F., Whitelegge J.P., Faull K., Reddy S., Farias-Eisner R. (2005) Characterization of serum biomarkers for detection of early stage ovarian cancer. *Proteomics* 5:4589-96. DOI: 10.1002/pmic.200500093.

Kozak K.R., Amneus M.W., Pusey S.M., Su F., Luong M.N., Luong S.A., Reddy S.T., Farias-Eisner R. (2003a) Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: Potential use in diagnosis and prognosis. *Proceedings of the National Academy of Sciences of the United States of America* 100:12343-12348. DOI: DOI 10.1073/pnas.2033602100.

Kozak K.R., Amneus M.W., Pusey S.M., Su F., Luong M.N., Luong S.A., Reddy S.T., Farias-Eisner R. (2003b) Identification of biomarkers for ovarian cancer using strong anion-

exchange ProteinChips: potential use in diagnosis and prognosis. *Proc Natl Acad Sci U S A* 100:12343-8. DOI: 10.1073/pnas.2033602100 2033602100 [pii].

Kristina G., Radomir P., Eva B., Lenka D., Radek L., Rostislav V., Borivoj V., Dalibor V. (2009) When one chip is not enough: Augmenting the validity of SELDI-TOF proteomic profiles of clinical specimens. *Lab on a Chip* 9:1014-1017. DOI: Doi 10.1039/B815503h.

Labussiere M., Idhah A., Wang X.W., Marie Y., Boisselier B., Falet C., Paris S., Laffaire J., Carpentier C., Criniere E., Ducray F., El Hallani S., Mokhtari K., Hoang-Xuan K., Delattre J.Y., Sanson M. (2010) All the 1p19q codeleted gliomas are mutated on IDH1 or IDH2. *Neurology* 74:1886-90. DOI: WNL.0b013e3181e1cf3a [pii] 10.1212/WNL.0b013e3181e1cf3a.

Lacroix M., Abi-Said D., Fournay D.R., Gokaslan Z.L., Shi W., DeMonte F., Lang F.F., McCutcheon I.E., Hassenbusch S.J., Holland E., Hess K., Michael C., Miller D., Sawaya R. (2001) A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *Journal of Neurosurgery* 95:190-8. DOI: 10.3171/jns.2001.95.2.0190.

Lai A., Tran A., Nghiemphu P.L., Pope W.B., Solis O.E., Selch M., Filka E., Yong W.H., Mischel P.S., Liau L.M., Phuphanich S., Black K., Peak S., Green R.M., Spier C.E., Kolevska T., Polikoff J., Fehrenbacher L., Elashoff R., Cloughesy T. (2011) Phase II Study of Bevacizumab Plus Temozolomide During and After Radiation Therapy for Patients With Newly Diagnosed Glioblastoma Multiforme. *Journal of Clinical Oncology* 29:142-148. DOI: Doi 10.1200/Jco.2010.30.2729.

Laperriere N., Zuraw L., Cairncross G., Guideli C.C.O.P. (2002) Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review. *Radiotherapy and Oncology* 64:259-273.

Larsen M., Tazzyman S., Lund E.L., Junker N., Lewis C.E., Kristjansen P.E.G., Murdoch C. (2008) Hypoxia-induced secretion of macrophage migration-inhibitory factor from MCF-7 breast cancer cells is regulated in a hypoxia-inducible factor-independent manner. *Cancer Letters* 265:239-249. DOI: DOI 10.1016/j.canlet.2008.02.012.

Latterich M., Abramovitz M., Leyland-Jones B. (2008) Proteomics: New technologies and clinical applications. *European Journal of Cancer* 44:2737-2741. DOI: DOI 10.1016/j.ejca.2008.09.007.

Lee M.E., Dyer D.H., Klein O.D., Bolduc J.M., Stoddard B.L., Koshland D.E. (1995) Mutational Analysis of the Catalytic Residues Lysine-230 and Tyrosine-160 in the NADP(+)-Dependent Isocitrate Dehydrogenase from *Escherichia-Coli*. *Biochemistry* 34:378-384.

Lees-Miller S.P., Godbout R., Chan D.W., Weinfeld M., Day R.S., Barron G.M., Allalunisturner J. (1995) Absence of P350 Subunit of DNA-Activated Protein-Kinase from a Radiosensitive Human Cell-Line. *Science* 267:1183-1185.

- Legendre H., Decaestecker C., Nagy N., Hendlisz A., Schuring M.P., Salmon I., Gabius H.J., Pector J.C., Kiss R. (2003) Prognostic values of galectin-3 and the macrophage migration inhibitory factor (MIF) in human colorectal cancers. *Modern Pathology* 16:491-504. DOI: Doi 10.1097/01.Mp.0000068235.45178.C1.
- Leger O., Johnsonleger C., Jackson E., Coles B., Dean C. (1994) The Chondroitin Sulfate Proteoglycan Ng2 Is a Tumor-Specific Antigen on the Chemically-Induced Rat Chondrosarcoma Hsn. *International Journal of Cancer* 58:700-705.
- Lei L., Wang X.J., Zheng Z.G., Huang J., Cao W.M., Chen Z.H., Shao X.Y., Cai J.F., Ye W.W., Lu H.Y. (2011) Identification of Serum Protein Markers for Breast Cancer Relapse With SELDI-TOF MS. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology* 294:941-944. DOI: Doi 10.1002/Ar.21399.
- Leng L., Bucala R. (2005) Macrophage migration inhibitory factor. *Critical Care Medicine* 33:S475-S477. DOI: Doi 10.1097/01.Ccm.0000191278.04636.D8.
- Lewczuk P., Esselmann H., Groemer T.W., Bibl M., Maler J.M., Steinacker P., Otto M., Kornhuber J., Wiltfang J. (2004) Amyloid beta peptides in cerebrospinal fluid as profiled with surface enhanced laser desorption/ionization time-of-flight mass spectrometry: Evidence of novel biomarkers in Alzheimer's disease. *Biological Psychiatry* 55:524-530. DOI: DOI 10.1016/j.biopsych.2003.10.014.
- Li J., Zhao J., Yu X., Lange J., Kuerer H., Krishnamurthy S., Schilling E., Khan S.A., Sukumar S., Chan D.W. (2005a) Identification of biomarkers for breast cancer in nipple aspiration and ductal lavage fluid. *Clin Cancer Res* 11:8312-20. DOI: 11/23/8312 [pii] 10.1158/1078-0432.CCR-05-1538.
- Li J.N., Zhang Z., Rosenzweig J., Wang Y.Y., Chan D.W. (2002) Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clinical Chemistry* 48:1296-1304.
- Li J.N., Zhao J., Yu X.D., Lange J., Kuerer H., Krishnamurthy S., Schilling E., Khan S.A., Sukumar S., Chan D.W. (2005b) Identification of biomarkers for breast cancer in nipple aspiration and ductal lavage fluid. *Clinical Cancer Research* 11:8312-8320. DOI: Doi 10.1158/1078-0432.Ccr-05-1538.
- Li L.H., Xie T. (2005) Stem cell niche: Structure and function. *Annual Review of Cell and Developmental Biology* 21:605-631. DOI: DOI 10.1146/annurev.cellbio.21.012704.131525.
- Li L.H., Neaves W.B. (2006) Normal stem cells and cancer stem cells: The niche matters. *Cancer Research* 66:4553-4557. DOI: Doi 10.1158/0008-5472.Can-05-3986.
- Li L.N., Quang T.S., Gracely E.J., Kim J.H., Emrich J.G., Yaeger T.E., Jenrette J.M., Cohen S.C., Black P., Brady L.W. (2010) A Phase II study of anti-epidermal growth factor receptor radioimmunotherapy in the treatment of glioblastoma multiforme Clinical article. *Journal of Neurosurgery* 113:192-198. DOI: Doi 10.3171/2010.2.Jns091211.

- Li Z.Z., Wang H., Eyler C.E., Hjeimeia A.B., Rich J.N. (2009) Turning Cancer Stem Cells Inside Out: An Exploration of Glioma Stem Cell Signaling Pathways. *Journal of Biological Chemistry* 284:16705-1609. DOI: DOI 10.1074/jbc.R900013200.
- Lin, J.-C., S.-Y. Chang, D.-S. Hsieh, C.-F. Lee, and D.-S. Yu. 2005. The association of Id-1, MIF and GSTpi with acquired drug resistance in hormone independent prostate cancer cells. *Oncology Reports* 13:983-8.
- Lin Y.M., Jan H.J., Lee C.C., Tao H.Y., Shih Y.L., Wei H.W., Lee H.M. (2008) Dexamethasone reduced invasiveness of human malignant glioblastoma cells through a MAPK phosphatase-1 (MKP-1) dependent mechanism. *European Journal of Pharmacology* 593:1-9. DOI: DOI 10.1016/j.ejphar.2008.06.111.
- Lin Z., Jenson S.D., Lim M.S., Elenitoba-Johnson K.S., Lin Z., Jenson S.D., Lim M.S., Elenitoba-Johnson K.S.J. (2004) Application of SELDI-TOF mass spectrometry for the identification of differentially expressed proteins in transformed follicular lymphoma. *Modern Pathology* 17:670-8.
- Lindsley J.E., Wang J.C. (1991) Proteolysis Patterns of Epitopically Labeled Yeast DNA Topoisomerase-Ii Suggest an Allosteric Transition in the Enzyme Induced by Atp Binding. *Proceedings of the National Academy of Sciences of the United States of America* 88:10485-10489.
- Lindsley J.E., Wang J.C. (1993) On the Coupling between Atp Usage and DNA Transport by Yeast DNA Topoisomerase-Ii. *Journal of Biological Chemistry* 268:8096-8104.
- Liu C., Sage J.C., Miller M.R., Verhaak R.G.W., Hippenmeyer S., Vogel H., Foreman O., Bronson R.T., Nishiyama A., Luo L.Q., Zong H. (2011) Mosaic Analysis with Double Markers Reveals Tumor Cell of Origin in Glioma. *Cell* 146:209-221. DOI: DOI 10.1016/j.cell.2011.06.014.
- Liu C.B. (2011) The Application of SELDI-TOF-MS in Clinical Diagnosis of Cancers. *Journal of Biomedicine and Biotechnology*. DOI: Artn 245821 Doi 10.1155/2011/245821.
- Liu D.R., Cao L.P., Yu J.K., Que R.S., Jiang W.Z., Zhou Y.M., Zhu L.H. (2009) Diagnosis of Pancreatic Adenocarcinoma Using Protein Chip Technology. *Pancreatology* 9:127-135. DOI: Doi 10.1159/000178883.
- Liu J., Zheng S., Yu J.K., Zhang J.M., Chen Z., Liu J., Zheng S., Yu J.-k., Zhang J.-m., Chen Z. (2005) Serum protein fingerprinting coupled with artificial neural network distinguishes glioma from healthy population or brain benign tumor. *Journal of Zhejiang University Science B* 6:4-10.
- Llaguno S.A., Chen J., Kwon C.H., Jackson E.L., Li Y., Burns D.K., Alvarez-Buylla A., Parada L.F. (2009) Malignant Astrocytomas Originate from Neural Stem/Progenitor Cells in a Somatic Tumor Suppressor Mouse Model (vol 15, pg 45, 2009). *Cancer Cell* 15:240-240. DOI: DOI 10.1016/j.ccr.2009.02.008.
- Lomonaco S.L., Finniss S., Xiang C.L., Lee H.K., Jiang W., Lemke N., Rempel S.A., Mikkelsen T., Brodie C. (2011) Cilengitide induces autophagy-mediated cell death in glioma cells. *Neuro-Oncology* 13:857-865. DOI: DOI 10.1093/neuonc/nor073.

- Louis D.N., Ohgaki H., Wiestler O.D., Cavenee W.K., Burger P.C., Jouvét A., Scheithauer B.W., Kleihues P., Louis D.N., Ohgaki H., Wiestler O.D., Cavenee W.K., Burger P.C., Jouvét A., Scheithauer B.W., Kleihues P. (2007) The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica* 114:97-109.
- Lubec G., Afjehi-Sadat L. (2007) Limitations and pitfalls in protein identification by mass spectrometry. *Chemical Reviews* 107:3568-3584. DOI: Doi 10.1021/Cr068213f.
- Lukashev M.E., Werb Z. (1998) ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends in Cell Biology* 8:437-441.
- Lundy F.T., Orr D.F., Gallagher J.R., Maxwell P., Shaw C., Napier S.S., Cowan C.G., Lamey P.J., Marley J.J. (2004) Identification and overexpression of human neutrophil alpha-defensins (human neutrophil peptides 1, 2 and 3) in squamous cell carcinomas of the human tongue. *Oral Oncology* 40:139-144. DOI: Doi 10.1016/S1368-8375(03)00142-8.
- Maas C., de Vries E., Tait S.W., Borst J. (2011) Bid can mediate a pro-apoptotic response to etoposide and ionizing radiation without cleavage in its unstructured loop and in the absence of p53. *Oncogene* 30:3636-47. DOI: 10.1038/onc.2011.75 onc201175 [pii].
- MacBeath G. (2002) Protein microarrays and proteomics. *Nature Genetics* 32:526-532. DOI: Doi 10.1038/Ng1037.
- MacDonald T.J., Taga T., Shimada H., Tabrizi P., Zlokovic B.V., Cheresh D.A., Laug W.E. (2001) Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha v integrin antagonist. *Neurosurgery* 48:151-157.
- MacGrogan G., Rudolph P., Mascarel Id I., Mauriac L., Durand M., Avril A., Dilhuydy J.M., Robert J., Mathoulin-Pelissier S., Picot V., Floquet A., Sierankowski G., Coindre J.M., Mascarel Id I.d. (2003) DNA topoisomerase IIalpha expression and the response to primary chemotherapy in breast cancer. *British Journal of Cancer* 89:666-71.
- Mahesparan R., Read T.A., Lund-Johansen M., Skaftnesmo K.O., Bjerkvig R., Engebraaten O. (2003) Expression of extracellular matrix components in a highly infiltrative in vivo glioma model. *Acta Neuropathologica* 105:49-57. DOI: DOI 10.1007/s00401-002-0610-0.
- Majumdar M., Vuori K., Stallcup W.B. (2003) Engagement of the NG2 proteoglycan triggers cell spreading via rac and p130cas. *Cellular Signalling* 15:79-84.
- Makagiansar I.T., Williams S., Mustelin T., Stallcup W.B. (2007) Differential phosphorylation of NG2 proteoglycan by ERK and PKC alpha helps balance cell proliferation and migration. *Journal of Cell Biology* 178:155-165. DOI: DOI 10.1083/jcb.200612084.
- Makagiansar I.T., Williams S., Dahlin-Huppe K., Fukushi J., Mustelin T., Stallcup W.B. (2004) Phosphorylation of NG2 proteoglycan by protein kinase C-alpha regulates polarized membrane distribution and cell motility. *Journal of Biological Chemistry* 279:55262-55270. DOI: DOI 10.1074/jbc.M411045200.

- Malmer B., Gronberg H., Bergenheim A.T., Lenner P., Henriksson R. (1999) Familial aggregation of astrocytoma in northern Sweden: An epidemiological cohort study. *International Journal of Cancer* 81:366-370.
- Mann M., Jensen O.N. (2003) Proteomic analysis of post-translational modifications. *Nature Biotechnology* 21:255-261. DOI: Doi 10.1038/Nbt0303-255.
- Mano M.S., Rosa D.D., De Azambuja E., Ismael G.F., Durbecq V. (2007) The 17q12-q21 amplicon: Her2 and topoisomerase-IIalpha and their importance to the biology of solid tumours. *Cancer Treat Rev* 33:64-77. DOI: S0305-7372(06)00187-3 [pii] 10.1016/j.ctrv.2006.10.001.
- Mao Y., Sun M., Desai S.D., Liu L.F. (2000) SUMO-1 conjugation to topoisomerase I: A possible repair response to topoisomerase-mediated DNA damage. *Proc Natl Acad Sci U S A* 97:4046-51. DOI: 10.1073/pnas.080536597 080536597 [pii].
- Mao Y., Desai S.D., Ting C.Y., Hwang J., Liu L.F. (2001) 26 S proteasome-mediated degradation of topoisomerase II cleavable complexes. *Journal of Biological Chemistry* 276:40652-8.
- Masters J.R.W. (2000) Human cancer cell lines: fact and fantasy. *Nature Reviews Molecular Cell Biology* 1:233-236.
- Matsumoto Y., Kunishio K., Nagao S. (1999) Increased phosphorylation of DNA topoisomerase II in etoposide resistant mutants of human glioma cell line. *Journal of Neuro-Oncology* 45:37-46.
- Matsumoto Y., Tamiya T., Nagao S. (2005) Resistance to topoisomerase II inhibitors in human glioma cell lines overexpressing multidrug resistant associated protein (MRP) 2. *J Med Invest* 52:41-8.
- Maubant S., Saint-Dizier D., Boutillon M., Perron-Sierra F., Casara P.J., Hickman J.A., Tucker G.C., Van Obberghen-Schilling E. (2006) Blockade of alpha v beta3 and alpha v beta5 integrins by RGD mimetics induces anoikis and not integrin-mediated death in human endothelial cells. *Blood* 108:3035-44. DOI: blood-2006-05-023580 [pii] 10.1182/blood-2006-05-023580.
- Maurer G.D., Tritschler I., Adams B., Tabatabai G., Wick W., Stupp R., Weller M. (2009) Cilengitide modulates attachment and viability of human glioma cells, but not sensitivity to irradiation or temozolomide in vitro. *Neuro Oncol* 11:747-56. DOI: 15228517-2009-012 [pii] 10.1215/15228517-2009-012 [doi].
- Maurya P., Meleady P., Dowling P., Clynes M. (2007) Proteomic approaches for serum biomarker discovery in cancer. *Anticancer Research* 27:1247-1255.

- McClendon A.K., Rodriguez A.C., Osheroff N. (2005) Human topoisomerase II alpha rapidly relaxes positively supercoiled DNA - Implications for enzyme action ahead of replication forks. *Journal of Biological Chemistry* 280:39337-39345.
- McClendon A.K., Osheroff N., McClendon A.K., Osheroff N. (2007) DNA topoisomerase II, genotoxicity, and cancer. *Mutation Research* 623:83-97.
- McComb M.E., Perlman D.H., Huang H., Costello C.E. (2007) Evaluation of an on-target sample preparation system for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in conjunction with normal-flow peptide high-performance liquid chromatography for peptide mass fingerprint analyses. *Rapid Communications in Mass Spectrometry* 21:44-58. DOI: Doi 10.1002/Rcm.2805.
- McCombie G., Knochennuss R. (2004) Small-molecule MALDI using the matrix suppression effect to reduce or eliminate matrix background interferences. *Analytical Chemistry* 76:4990-4997. DOI: Doi 10.1021/Ac049581r.
- McLean M.H., Murray G.I., Stewart K.N., Norrie G., Mayer C., Hold G.L., Thomson J., Fyfe N., Hope M., Mowat N.A.G., Drew J.E., El-Omar E.M. (2011) The Inflammatory Microenvironment in Colorectal Neoplasia. *Plos One* 6. DOI: ARTN e15366 DOI 10.1371/journal.pone.0015366.
- Meikrantz W., Bergom M.A., Memisoglu A., Samson L. (1998) O6-alkylguanine DNA lesions trigger apoptosis. *Carcinogenesis* 19:369-72.
- Meister A. (1988) Glutathione Metabolism and Its Selective Modification. *Journal of Biological Chemistry* 263:17205-17208.
- Mellai M., Caldera V., Annovazzi L., Chio A., Lanotte M., Cassoni P., Finocchiaro G., Schiffer D. (2009) MGMT promoter hypermethylation in a series of 104 glioblastomas. *Cancer Genomics Proteomics* 6:219-27. DOI: 6/4/219 [pii].
- Merchant M., Weinberger S.R. (2000) Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis* 21:1164-1177.
- Meyer-Siegler K.L., Bellino M.A., Tannenbaum M. (2002) Macrophage migration inhibitory factor evaluation compared with prostate specific antigen as a biomarker in patients with prostate carcinoma. *Cancer* 94:1449-1456.
- Mikeska T., Bock C., El-Maarri O., Hubner A., Ehrentraut D., Schramm J., Felsberg J., Kahl P., Buttner R., Pietsch T., Waha A. (2007) Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *Journal of Molecular Diagnostics* 9:368-381. DOI: DOI 10.2353/jmoldx.2007.060167.
- Minden J.S., Dowd S.R., Meyer H.E., Stuhler K. (2009) Difference gel electrophoresis. *Electrophoresis* 30:S156-S161. DOI: DOI 10.1002/elps.200900098.
- Mischel P.S., Cloughesy T.F. (2003) Targeted molecular therapy of GBM. *Brain Pathology* 13:52-61.

- Mittelbronn M., Platten M., Zeiner P., Dombrowski Y., Frank B., Zachskorn C., Harter P.N., Weller M., Wischhusen J. (2011) Macrophage migration inhibitory factor (MIF) expression in human malignant gliomas contributes to immune escape and tumour progression. *Acta Neuropathologica* 122:353-365. DOI: DOI 10.1007/s00401-011-0858-3.
- Miyamae T., Malehorn D.E., Lemster B., Mori M., Imagawa T., Yokota S., Bigbee W.L., Welsh M., Klarskov K., Nishimoto N., Vallejo A.N., Hirsch R. (2005) Serum protein profile in systemic-onset juvenile idiopathic arthritis differentiates response versus nonresponse to therapy. *Arthritis Research & Therapy* 7:R746-R755. DOI: Doi 10.1186/Ar1723.
- Mohri M., Nitta H., Yamashita J. (2000) Expression of multidrug resistance-associated protein (MRP) in human gliomas. *Journal of Neuro-Oncology* 49:105-115.
- Mollemann M., Wolter M., Felsberg J., Collins V.P., Reifemberger G. (2005) Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. *International Journal of Cancer* 113:379-385. DOI: Doi 10.1002/Ijc.20575.
- Molloy M.P., Donohoe S., Brzezinski E.E., Kilby G.W., Stevenson T.I., Baker J.D., Goodlett D.R., Gage D.A. (2005) Large-scale evaluation of quantitative reproducibility and proteome coverage using acid cleavable isotope coded affinity tag mass spectrometry for proteomic profiling. *Proteomics* 5:1204-1208. DOI: DOI 10.1002/pmic.200400994.
- Momand J., Zambetti G.P., Olson D.C., George D., Levine A.J. (1992) The Mdm-2 Oncogene Product Forms a Complex with the P53 Protein and Inhibits P53-Mediated Transactivation. *Cell* 69:1237-1245.
- Montecucco A., Biamonti G., Montecucco A., Biamonti G. (2007) Cellular response to etoposide treatment. *Cancer Letters* 252:9-18.
- Moore B.W. (1965) A Soluble Protein Characteristic of Nervous System. *Biochemical and Biophysical Research Communications* 19:739-&.
- Muller C.A., Markovic-Lipkovski J., Klatt T., Gamper J., Schwarz G., Beck H., Deeg M., Kalbacher H., Widmann S., Wessels J.T., Becker V., Muller G.A., Flad T. (2002) Human alpha-defensins HNPs-1,-2, and-3 in renal cell carcinoma - Influences on tumor cell proliferation. *American Journal of Pathology* 160:1311-1324.
- Munaut C., Boniver J., Foidart J.M., Deprez M. (2002) Macrophage migration inhibitory factor (MIF) expression in human glioblastomas correlates with vascular endothelial growth factor (VEGF) expression. *Neuropathology and Applied Neurobiology* 28:452-460.
- Muss H.B., Thor A.D., Berry D.A., Kute T., Liu E.T., Koerner F., Cirrincione C.T., Budman D.R., Wood W.C., Barcos M., Henderson I.C. (1994) C-ErbB-2 Expression and Response to Adjuvant Therapy in Women with Node-Positive Early Breast-Cancer. *New England Journal of Medicine* 330:1260-1266.
- Nakaji P., Gore P., Spetzler R.F. (2005) Management of arteriovenous malformations: A surgical perspective. *Neurology India* 53:14-16.

- Nakamura M., Watanabe T., Yonekawa Y., Kleihues P., Ohgaki H. (2001) Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G : C -> A : T mutations of the TP53 tumor suppressor gene. *Carcinogenesis* 22:1715-1719.
- Nakatsu Y., Hattori K., Hayakawa H., Shimizu K., Sekiguchi M. (1993) Organization and Expression of the Human Gene for O6-Methylguanine-DNA Methyltransferase. *Mutation Research* 293:119-132.
- Natarajan A.T., Vermeulen S., Darroudi F., Valentine M.B., Brent T.P., Mitra S., Tano K. (1992) Chromosomal Localization of Human O-6-Methylguanine-DNA Methyltransferase (Mgmt) Gene by Insitu Hybridization. *Mutagenesis* 7:83-85.
- Neufeld G., Cohen T., Gengrinovitch S., Poltorak Z. (1999) Vascular endothelial growth factor (VEGF) and its receptors. *Faseb Journal* 13:9-22.
- Newlands E.S., O'Reilly S.M., Glaser M.G., Bower M., Evans H., Brock C., Brampton M.H., Colquhoun I., Lewis P., Rice-Edwards J.M., Illingworth R.D., Richards P.G. (1996) The Charing Cross Hospital experience with temozolomide in patients with gliomas. *European Journal of Cancer* 32A:2236-2241.
- Niclou S.P., Fack F., Rajcevic U. (2010) Glioma proteomics: Status and perspectives. *Journal of Proteomics* In Press, Corrected Proof.
- Nieder C., Grosu A.L., Astner S., Molls M. (2005) Treatment of unresectable glioblastoma multiforme. *Anticancer Research* 25:4605-4610.
- Nishiyama A., Dahlin K.J., Stallcup W.B. (1991a) The Expression of Ng2 Proteoglycan in the Developing Rat Limb. *Development* 111:933-944.
- Nishiyama A., Lin X.H., Stallcup W.B. (1995) Generation of Truncated Forms of the Ng2 Proteoglycan by Cell-Surface Proteolysis. *Molecular Biology of the Cell* 6:1819-1832.
- Nishiyama A., Dahlin K.J., Prince J.T., Johnston S.R., Stallcup W.B. (1991b) The Primary Structure of Ng2, a Novel Membrane-Spanning Proteoglycan. *Journal of Cell Biology* 114:359-371.
- Nobusawa S., Lachuer J., Wierinckx A., Kim Y.H., Huang J.A., Legras C., Kleihues P., Ohgaki H. (2010) Intratumoral Patterns of Genomic Imbalance in Glioblastomas. *Brain Pathology* 20:936-944. DOI: 10.1111/j.1750-3639.2010.00395.x.
- Nomiya T., Nemoto K., Kumabe T., Takai Y., Yamada S. (2007) Prognostic significance of surgery and radiation therapy in cases of anaplastic astrocytoma: retrospective analysis of 170 cases. *Journal of Neurosurgery* 106:575-581.
- Oakman C., Moretti E., Galardi F., Santarpia L., Di Leo A. (2009) The role of topoisomerase IIalpha and HER-2 in predicting sensitivity to anthracyclines in breast cancer patients. *Cancer Treat Rev* 35:662-7. DOI: S0305-7372(09)00121-2 [pii] 10.1016/j.ctrv.2009.08.006.

- Ohgaki H., Kleihues P. (2005) Epidemiology and etiology of gliomas. *Acta Neuropathologica* 109:93-108. DOI: 10.1007/s00401-005-0991-y.
- Ohgaki H., Kleihues P., Ohgaki H., Kleihues P. (2007) Genetic pathways to primary and secondary glioblastoma. *American Journal of Pathology* 170:1445-53.
- Ohgaki H., Dessen P., Jourde B., Horstmann S., Nishikawa T., Di Patre P.L., Burkhard C., Schuler D., Probst-Hensch N.M., Maiorka P.C., Baeza N., Pisani P., Yonekawa Y., Yasargil M.G., Lutolf U.M., Kleihues P., Ohgaki H., Dessen P., Jourde B., Horstmann S., Nishikawa T., Di Patre P.-L., Burkhard C., Schuler D., Probst-Hensch N.M., Maiorka P.C., Baeza N., Pisani P., Yonekawa Y., Yasargil M.G., Lutolf U.M., Kleihues P. (2004) Genetic pathways to glioblastoma: a population-based study. *Cancer Research* 64:6892-9.
- Oldenhuis C.N.A.M., Oosting S.F., Gietema J.A., de Vries E.G.E. (2008) Prognostic versus predictive value of biomarkers in oncology. *European Journal of Cancer* 44:946-953. DOI: DOI 10.1016/j.ejca.2008.03.006.
- Oliveira-Ferrer L., Hauschild J., Fiedler W., Bokemeyer C., Nippgen J., Celik I., Schuch G. (2008) Cilengitide induces cellular detachment and apoptosis in endothelial and glioma cells mediated by inhibition of FAK/src/AKT pathway. *Journal of Experimental & Clinical Cancer Research* 27:86.
- Oreilly S.M., Newlands E.S., Glaser M.G., Brampton M., Riceedwards J.M., Illingworth R.D., Richards P.G., Kennard C., Colquhoun I.R., Lewis P., Stevens M.F.G. (1993) Temozolomide - a New Oral Cytotoxic Chemotherapeutic Agent with Promising Activity against Primary Brain-Tumors. *European Journal of Cancer* 29A:940-942.
- Osheroff N. (1987) Role of the Divalent-Cation in Topoisomerase-Ii Mediated Reactions. *Biochemistry* 26:6402-6406.
- Ott H.W., Lindner H., Sarg B., Mueller-Holzner E., Abendstein B., Bergant A., Fessler S., Schwaerzler P., Zeimet A., Marth C., Illmensee K. (2003) Calgranulins in cystic fluid and serum from patients with ovarian carcinomas. *Cancer Research* 63:7507-7514.
- Ozerdem U., Stallcup W.B. (2003) Early contribution of pericytes to angiogenic sprouting and tube formation. *Angiogenesis* 6:241-9. DOI: 10.1023/B:AGEN.0000021401.58039.a9 5265361 [pii].
- Ozerdem U., Monosov E., Stallcup W.B. (2002) NG2 proteoglycan expression by pericytes in pathological microvasculature. *Microvascular Research* 63:129-134. DOI: DOI 10.1006/mvre.2001.2376.
- Ozerdem U., Grako K.A., Dahlin-Huppe K., Monosov E., Stallcup W.B. (2001) NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Developmental Dynamics* 222:218-227.
- Pan Y.Z., Xiao X.Y., Zhao D., Zhang L., Ji G.Y., Li Y., Yang B.X., He D.C., Zhao X.J. (2006) Application of surface-enhanced laser desorption/ionization time-of-flight-based serum

proteomic array technique for the early diagnosis of prostate cancer. *Asian Journal of Andrology* 8:45-51. DOI: DOI 10.1111/j.1745-7262.2006.00103.x.

Panicker G., Lee D.R., Unger E.R., Panicker G., Lee D.R., Unger E.R. (2009a) Optimization of SELDI-TOF protein profiling for analysis of cervical mucous. *Journal of Proteomics* 71:637-46.

Panicker G.K., Kerkar V., Natekar M., Karnad D.R., Kothari S., Narula O. (2009b) Subjects in a Positive Thorough Qt Study Respond Similarly to Moxifloxacin and Two Doses of Study Drug: Is This a Pharmacokinetic or a Pharmacodynamic Response? *Journal of Clinical Pharmacology* 49:1112-1112.

Papale M., Pedicillo M.C., Thatcher B.J., Di Paolo S., Lo Muzio L., Bufo P., Rocchetti M.T., Centra M., Ranieri E., Gesualdo L. (2007) Urine profiling by SELDI-TOF/MS: Monitoring of the critical steps in sample collection, handling and analysis. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 856:205-213. DOI: DOI 10.1016/j.jchromb.2007.06.001.

Pardridge W.M. (2005) The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* 2:3-14. DOI: 10.1602/neurorx.2.1.3.

Parker C.E., Pearson T.W., Anderson N.L., Borchers C.H. (2010) Mass-spectrometry-based clinical proteomics - a review and prospective. *Analyst* 135:1830-1838. DOI: Doi 10.1039/C0an00105h.

Parsons D.W., Jones S., Zhang X.S., Lin J.C.H., Leary R.J., Angenendt P., Mankoo P., Carter H., Siu I.M., Gallia G.L., Olivi A., McLendon R., Rasheed B.A., Keir S., Nikolskaya T., Nikolsky Y., Busam D.A., Tekleab H., Diaz L.A., Hartigan J., Smith D.R., Strausberg R.L., Marie S.K.N., Shinjo S.M.O., Yan H., Riggins G.J., Bigner D.D., Karchin R., Papadopoulos N., Parmigiani G., Vogelstein B., Velculescu V.E., Kinzler K.W. (2008) An integrated genomic analysis of human glioblastoma Multiforme. *Science* 321:1807-1812. DOI: DOI 10.1126/science.1164382.

Patel S., Breneman J.C., Warnick R.E., Albright R.E., Tobler W.D., van Loveren H.R., Tew J.M. (2000) Permanent iodine-125 interstitial implants for the treatment of recurrent glioblastoma multiforme. *Neurosurgery* 46:1123-1128.

Patra N., De U., Kang J.A., Kim J.M., Ahn M.Y., Lee J., Jung J.H., Chung H.Y., Moon H.R., Kim H.S. (2011) A novel epoxypropoxy flavonoid derivative and topoisomerase II inhibitor, MHY336, induces apoptosis in prostate cancer cells. *European Journal of Pharmacology* 658:98-107. DOI: 10.1016/j.ejphar.2011.02.015.

Payne M.J., Pratap S.E., Middleton M.R. (2005) Temozolomide in the treatment of solid tumours: current results and rationale for dosing/scheduling. *Critical Reviews in Oncology Hematology* 53:241-252. DOI: DOI 10.1016/j.critevonc.2004.10.004.

Paz M.F., Yaya-Tur R., Rojas-Marcos I., Reynes G., Pollan M., Aguirre-Cruz L., Garcia-Lopez J.L., Piquer J., Safont M.J., Balana C., Sanchez-Cespedes M., Garcia-Villanueva M., Arribas L., Esteller M. (2004) CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. *Clinical Cancer Research* 10:4933-4938.

- Pegg A.E. (2000) Repair of O-6-alkylguanine by alkyltransferases. *Mutation Research-Reviews in Mutation Research* 462:83-100.
- Pegg A.E., Dolan M.E., Moschel R.C. (1995) Structure, Function, and Inhibition of O-6-Alkylguanine-DNA Alkyltransferase. *Progress in Nucleic Acid Research and Molecular Biology*, Vol 51 51:167-223.
- Peng J.M., Gygi S.P. (2001) Proteomics: the move to mixtures. *Journal of Mass Spectrometry* 36:1083-1091.
- Persson A.I., Petritsch C., Swartling F.J., Itsara M., Sim F.J., Auvergne R., Goldenberg D.D., Vandenberg S.R., Nguyen K.N., Yakovenko S., Ayers-Ringler J., Nishiyama A., Stallcup W.B., Berger M.S., Bergers G., McKnight T.R., Goldman S.A., Weiss W.A. (2010) Non-Stem Cell Origin for Oligodendroglioma. *Cancer Cell* 18:669-682. DOI: DOI 10.1016/j.ccr.2010.10.033.
- Petricoin E.F., Ardekani A.M., Hitt B.A., Levine P.J., Fusaro V.A., Steinberg S.M., Mills G.B., Simone C., Fishman D.A., Kohn E.C., Liotta L.A. (2002a) Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359:572-577.
- Petricoin E.F., Ornstein D.K., Paweletz C.P., Ardekani A., Hackett P.S., Hitt B.A., Velasco A., Trucco C., Wiegand L., Wood K., Simone C.B., Levine P.J., Linehan W.M., Emmert-Buck M.R., Steinberg S.M., Kohn E.C., Liotta L.A. (2002b) Serum proteomic patterns for detection of prostate cancer. *Journal of the National Cancer Institute* 94:1576-1578.
- Petrik V., Saadoun S., Loosemore A., Hobbs J., Opstad K.S., Sheldon J., Tarelli E., Howe F.A., Bell B.A., Papadopoulos M.C. (2008) Serum alpha(2)-HS glycoprotein predicts survival in patients with glioblastoma. *Clinical Chemistry* 54:713-722. DOI: DOI 10.1373/clinchem.2007.096792.
- Phillips H.S., Kharbanda S., Chen R.H., Forrest W.F., Soriano R.H., Wu T.D., Misra A., Nigro J.M., Colman H., Soroceanu L., Williams P.M., Modrusan Z., Feuerstein B.G., Aldape K. (2006) Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 9:157-173. DOI: DOI 10.1016/j.ccr.2006.02.019.
- Piette C., Deprez M., Roger T., Noel A., Foidart J.M., Munaut C. (2009) The Dexamethasone-induced Inhibition of Proliferation, Migration, and Invasion in Glioma Cell Lines Is Antagonized by Macrophage Migration Inhibitory Factor (MIF) and Can Be Enhanced by Specific MIF Inhibitors. *Journal of Biological Chemistry* 284:32483-32492. DOI: DOI 10.1074/jbc.M109.014589.
- Plate K.H., Risau W. (1995) Angiogenesis in Malignant Gliomas. *Glia* 15:339-347.
- Polk D.B., Peek R.M. (2010) *Helicobacter pylori*: gastric cancer and beyond. *Nature Reviews Cancer* 10:403-414. DOI: Doi 10.1038/Nrc2857.
- Poon T.C., Yip T.T., Chan A.T., Yip C., Yip V., Mok T.S., Lee C.C., Leung T.W., Ho S.K., Johnson P.J., Poon T.C.W., Yip T.-T., Chan A.T.C., Yip C., Yip V., Mok T.S.K., Lee C.C.Y., Leung T.W.T., Ho S.K.W., Johnson P.J. (2003) Comprehensive proteomic

profiling identifies serum proteomic signatures for detection of hepatocellular carcinoma and its subtypes. *Clinical Chemistry* 49:752-60.

Poon T.C.W. (2007) Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. *Expert Review of Proteomics* 4:51-65. DOI: Doi 10.1586/14789450.4.1.51.

Popescu I.D., Albulescu R., Raducan E., Dinischiotu A., Tanase C. (2010) Applications of SELDI-TOF technology in cancer biomarkers discovery. *Romanian Biotechnological Letters* 15:5654-5667.

Pouliot J.J., Robertson C.A., Nash H.A. (2001) Pathways for repair of topoisomerase I covalent complexes in *Saccharomyces cerevisiae*. *Genes Cells* 6:677-87. DOI: gtc452 [pii].

Pouliot J.J., Yao K.C., Robertson C.A., Nash H.A. (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. *Science* 286:552-5. DOI: 7897 [pii].

Preusser M., de Ribaupierre S., Wohrer A., Erridge S.C., Hegi M., Weller M., Stupp R. (2011) Current Concepts and Management of Glioblastoma. *Annals of Neurology* 70:9-21. DOI: Doi 10.1002/Ana.22425.

Preusser M., Janzer R.C., Felsberg J., Reifenberger G., Hamou M.F., Diserens A.C., Stupp R., Gorlia T., Marosi C., Heinzl H., Hainfellner J.A., Hegi M. (2008) Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: Observer variability and lack of association with patient survival impede its use as clinical biomarker. *Brain Pathology* 18:520-532. DOI: DOI 10.1111/j.1750-3639.2008.00153.x.

Previtali S., Quattrini A., Nemni R., Truci G., Ducati A., Wrabetz L., Canal N. (1996) alpha 6 beta 4 and alpha 6 beta 1 Integrins in astrocytomas and other CNS tumors. *Journal of Neuropathology and Experimental Neurology* 55:456-465.

Price M.A., Wanshura L.E.C., Yang J.B., Carlson J., Xiang B., Li G.Y., Ferrone S., Dudek A.Z., Turley E.A., McCarthy J.B. (2011) CSPG4, a potential therapeutic target, facilitates malignant progression of melanoma. *Pigment Cell & Melanoma Research* 24:1148-1157. DOI: DOI 10.1111/j.1755-148X.2011.00929.x.

Pusztai L., Gregory B.W., Baggerly K.A., Peng B., Koomen J., Kuerer H.M., Esteva F.J., Symmans W.F., Wagner P., Hortobagyi G.N., Laronga C., Semmes O.J., Wright G.L., Drake R.R., Vlahou A. (2004) Pharmacoproteomic analysis of prechemotherapy and postchemotherapy plasma samples from patients receiving neoadjuvant or adjuvant chemotherapy for breast carcinoma. *Cancer* 100:1814-1822. DOI: Doi 10.1002/Cncr.20203.

Qu Y.S., Adam B.L., Yasui T., Ward M.D., Cazares L.H., Schellhammer P.F., Feng Z.D., Semmes O.J., Wright G.L. (2002) Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clinical Chemistry* 48:1835-1843.

- Ranganathan S., Williams E., Ganchev P., Gopalakrishnan V., Lacomis D., Urbinelli L., Newhall K., Cudkowicz M.E., Brown R.H., Bowser R. (2005) Proteomic profiling of cerebrospinal fluid identifies biomarkers for amyotrophic lateral sclerosis. *Journal of Neurochemistry* 95:1461-1471. DOI: DOI 10.1111/j.1471-4159.2005.03478.x.
- Reardon D.A., Fink K.L., Mikkelsen T., Cloughesy T.F., O'Neill A., Plotkin S., Glantz M., Ravin P., Raizer J.J., Rich K.M., Schiff D., Shapiro W.R., Burdette-Radoux S., Dropcho E.J., Wittmer S.M., Nippgen J., Picard M., Nabors L.B. (2008) Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme. *Journal of Clinical Oncology* 26:5610-7.
- Rein B.J., Gupta S., Dada R., Safi J., Michener C., Agarwal A. (2011) Potential markers for detection and monitoring of ovarian cancer. *J Oncol* 2011:475983. DOI: 10.1155/2011/475983.
- Reya T., Morrison S.J., Clarke M.F., Weissman I.L. (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414:105-111.
- Reynolds A.R., Hart I.R., Watson A.R., Welte J.C., Silva R.G., Robinson S.D., Da Violante G., Gourlaouen M., Salih M., Jones M.C., Jones D.T., Saunders G., Kostourou V., Perron-Sierra F., Norman J.C., Tucker G.C., Hodivala-Dilke K.M. (2009) Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nature Medicine* 15:392-400. DOI: Doi 10.1038/Nm.1941.
- Ricard D., Kaloshi G., Amiel-Benoualch A., Lejeune J., Marie Y., Mandonnet E., Kujas M., Mokhtari K., Taillibert S., Laigle-Donadey F., Carpentier A.F., Omuro A., Capelle L., Duffau H., Cornu P., Guillevin R., Sanson M., Hoang-Xuan K., Delattre J.Y. (2007) Dynamic history of low-grade gliomas before and after temozolomide treatment. *Annals of Neurology* 61:484-490. DOI: Doi 10.1002/Ana.21125.
- Rice W.G., Ganz T., Kinkade J.M., Selsted M.E., Lehrer R.I., Parmley R.T. (1987) Defensin-Rich Dense Granules of Human-Neutrophils. *Blood* 70:757-765.
- Rich J.N., Reardon D.A., Peery T., Dowell J.M., Quinn J.A., Penne K.L., Wikstrand C.J., Van Duyn L.B., Dancey J.E., McLendon R.E., Kao J.C., Stenzel T.T., Rasheed B.K.A., Tourt-Uhlig S.E., Herndon J.E., Vredenburgh J.J., Sampson J.H., Friedman A.H., Bigner D.D., Friedman H.S. (2004) Phase II trial of gefitinib in recurrent glioblastoma. *Journal of Clinical Oncology* 22:133-142. DOI: Doi 10.1200/Jco.2004.08.110.
- Riemenschneider M.J., Jeuken J.W.M., Wesseling P., Reifenberger G. (2010) Molecular diagnostics of gliomas: state of the art. *Acta Neuropathologica* 120:567-84.
- Riepl B., Grassel S., Wiest R., Fleck M., Straub R.H. (2010) Tumor necrosis factor and norepinephrine lower the levels of human neutrophil peptides 1-3 secretion by mixed synovial tissue cultures in osteoarthritis and rheumatoid arthritis. *Arthritis Research & Therapy* 12. DOI: Artn R110 Doi 10.1186/Ar3044.

- Righetti S.C., Perego P., Corna E., Pierotti M.A., Zunino F. (1999) Emergence of p53 mutant cisplatin-resistant ovarian carcinoma cells following drug exposure: spontaneously mutant selection. *Cell Growth Differ* 10:473-8.
- Rodriguez F.J., Thibodeau S.N., Jenkins R.B., Schowalter K.V., Caron B.L., O'Neill B.P., James C.D., Passe S., Slezak J., Giannini C. (2008) MGMT immunohistochemical expression and promoter methylation in human glioblastoma. *Applied Immunohistochemistry & Molecular Morphology* 16:59-65.
- Roesch-Ely M., Nees M., Karsai S., Ruess A., Bogumil R., Warnken U., Schnolzer M., Dietz A., Plinkert P.K., Hofele C., Bosch F.X. (2007) Proteomic analysis reveals successive aberrations in protein expression from healthy mucosa to invasive head and neck cancer. *Oncogene* 26:54-64. DOI: DOI 10.1038/sj.onc.1209770.
- Rosengren E., Bucala R., Aman P., Jacobsson L., Odh G., Metz C.N., Rorsman H. (1996) The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Molecular Medicine* 2:143-149.
- Ross P.L., Huang Y.L.N., Marchese J.N., Williamson B., Parker K., Hattan S., Khainovski N., Pillai S., Dey S., Daniels S., Purkayastha S., Juhasz P., Martin S., Bartlet-Jones M., He F., Jacobson A., Pappin D.J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Molecular & Cellular Proteomics* 3:1154-1169. DOI: DOI 10.1074/mcp.M400129-MCP200.
- Rossmann J., Reddy V., Cantor A., Miley D., Robert F. (2011) Phase II study of dose-intense chemotherapy with sequential topoisomerase-targeting regimens with irinotecan/oxaliplatin followed by etoposide/carboplatin in chemotherapy naive patients with extensive small cell lung cancer. *Lung Cancer* 72:219-223. DOI: 10.1016/j.lungcan.2010.08.023.
- Roth J., Vogl T., Sorg C., Sunderkotter C. (2003) Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends in Immunology* 24:155-158. DOI: Doi 10.1016/S1471-4906(03)00062-0.
- Rückert F., Pilarsky C., Grützmann R. (2010) Serum Tumor Markers in Pancreatic Cancer—Recent Discoveries. *Cancers* 2:1107-1124.
- Ruoslahti E. (1996) RGD and other recognition sequences for integrins. *Annual Review of Cell & Developmental Biology* 12:697-715.
- Sampson J.H., Archer G.E., Mitchell D.A., Heimberger A.B., Herndon J.E., Lally-Goss D., McGehee-Norman S., Paolino A., Reardon D.A., Friedman A.H., Friedman H.S., Bigner D.D. (2009) An epidermal growth factor receptor variant III-targeted vaccine is safe and immunogenic in patients with glioblastoma multiforme. *Molecular Cancer Therapeutics* 8:2773-2779. DOI: Doi 10.1158/1535-7163.Mct-09-0124.
- Sampson J.H., Heimberger A.B., Archer G.E., Aldape K.D., Friedman A.H., Friedman H.S., Gilbert M.R., Herndon J.E., McLendon R.E., Mitchell D.A., Reardon D.A., Sawaya R., Schmittling R.J., Shi W.M., Vredenburgh J.J., Bigner D.D. (2010) Immunologic Escape After Prolonged Progression-Free Survival With Epidermal Growth Factor Receptor

Variant III Peptide Vaccination in Patients With Newly Diagnosed Glioblastoma. *Journal of Clinical Oncology* 28:4722-4729. DOI: Doi 10.1200/Jco.2010.28.6963.

Sandri M.I., Isaacs R.J., Ongkeko W.M., Harris A.L., Hickson I.D., Broggini M., Vikhanskaya F. (1996) p53 regulates the minimal promoter of the human topoisomerase II alpha gene. *Nucleic Acids Research* 24:4464-4470.

Sandvig A., Berry M., Barrett L.B., Butt A., Logan A. (2004) Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: Expression, receptor signaling, and correlation with axon regeneration. *Glia* 46:225-251. DOI: Doi 10.1002/Glia.10315.

Sanson M., Marie Y., Paris S., Idbaih A., Laffaire J., Ducray F., El Hallani S., Boisselier B., Mokhtari K., Hoang-Xuan K., Delattre J.Y. (2009) Isocitrate Dehydrogenase 1 Codon 132 Mutation Is an Important Prognostic Biomarker in Gliomas. *Journal of Clinical Oncology* 27:4150-4154. DOI: Doi 10.1200/Jco.2009.21.9832.

Sauve A.C., Speed T.P. (2004) Normalisation, Baseline Correction and Alignment of High-Throughput Mass Spectrometry Data. <http://www.stat.berkeley.edu/~terry/Group/publications/Final2Gensips2004Sauve.pdf> Accessed in October 2011.

Sawaya R., Hammoud M., Schoppa D., Hess K.R., Wu S.Z., Shi W.M., Wildrick D.M. (1998) Neurosurgical outcomes in a modern series of 400 craniotomies for treatment of parenchymal tumors. *Neurosurgery* 42:1044-1055.

Scarlett C.J., Saxby A.J., Nielsen A., Bell C., Samra J.S., Hugh T., Baxter R.C., Smith R.C., Scarlett C.J., Saxby A.J., Nielsen A., Bell C., Samra J.S., Hugh T., Baxter R.C., Smith R.C. (2006) Proteomic profiling of cholangiocarcinoma: diagnostic potential of SELDI-TOF MS in malignant bile duct stricture. *Hepatology* 44:658-66.

Schafer B.W., Heizmann C.W. (1996) The S100 family of EF-hand calcium-binding proteins: Functions and pathology. *Trends in Biochemical Sciences* 21:134-140.

Schlingemann R.O., Rietveld F.J.R., Dewaal R.M.W., Ferrone S., Ruiter D.J. (1990) Expression of the High-Molecular-Weight Melanoma-Associated Antigen by Pericytes during Angiogenesis in Tumors and in Healing Wounds. *American Journal of Pathology* 136:1393-1405.

Schrapppe M., Klier F.G., Spiro R.C., Waltz T.A., Reisfeld R.A., Gladson C.L. (1991) Correlation of Chondroitin Sulfate Proteoglycan Expression on Proliferating Brain Capillary Endothelial-Cells with the Malignant Phenotype of Astroglial Cells. *Cancer Research* 51:4986-4993.

Schwartzbaum J., Ahlbom A., Malmer B., Lonn S., Brookes A.J., Doss H., Debinski W., Henriksson R., Feychting M. (2005) Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. *Cancer Research* 65:6459-6465.

Schwartzbaum J.A., Fisher J.L., Aldape K.D., Wrensch M. (2006) Epidemiology and molecular pathology of glioma. *Nature Clinical Practice Neurology* 2:494-503; quiz 1 p following 516.

- Scott J.N., Brasher P.M.A., Sevick R.J., Rewcastle N.B., Forsyth P.A. (2002) How often are nonenhancing supratentorial gliomas malignant? A population study. *Neurology* 59:947-949.
- Scrutton N.S., Berry A., Perham R.N. (1990) Redesign of the Coenzyme Specificity of a Dehydrogenase by Protein Engineering. *Nature* 343:38-43.
- Seibert V., Wiesner A., Buschmann T., Meuer J. (2004) Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI TOF-MS) and ProteinChip((R)) technology in proteomics research. *Pathology Research and Practice* 200:83-94. DOI: DOI 10.1016/j.prp.2004.01.010.
- Seidel S., Garvalov B.K., Wirta V., von Stechow L., Schanzer A., Meletis K., Wolter M., Sommerlad D., Henze A.T., Nister M., Reifemberger G., Lundeborg J., Frisen J., Acker T. (2010) A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain* 133:983-995. DOI: Doi 10.1093/Brain/Awq042.
- Selsted M.E., Ouellette A.J. (2005) Mammalian defensins in the antimicrobial immune response. *Nature Immunology* 6:551-557. DOI: Doi 10.1038/Ni1206.
- Sevim, H., J.F. Parkinson, and K.L. McDonald. 2011. Etoposide-mediated glioblastoma cell death: dependent or independent on the expression of its target, topoisomerase II alpha? *Journal of Cancer Research & Clinical Oncology* 137:1705-12.
- Sheta E.A., Appel S.H., Goldknopf I.L. (2006) 2D gel blood serum biomarkers reveal differential clinical proteomics of the neurodegenerative diseases. *Expert Review of Proteomics* 3:45-62. DOI: Doi 10.1586/14789450.3.1.45.
- Shimizu T., Abe R., Nakamura H., Ohkawara A., Suzuki M., Nishihira J. (1999) High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. *Biochemical and Biophysical Research Communications* 264:751-758.
- Shoshan Y., Nishiyama A., Chang A.S., Mork S., Barnett G.H., Cowell J.K., Trapp B.D., Staugaitis S.M. (1999) Expression of oligodendrocyte progenitor cell antigens by gliomas: Implications for the histogenesis of brain tumors. *Proceedings of the National Academy of Sciences of the United States of America* 96:10361-10366.
- Singh R.K., Varney M.L. (1998) Regulation of interleukin 8 expression in human malignant melanoma cells. *Cancer Research* 58:1532-1537.
- Slotman B.J., Kralendonk J.H., van Alphen H.A., Kamphorst W., Karim A.B. (1996) Hypofractionated radiation therapy in patients with glioblastoma multiforme: results of treatment and impact of prognostic factors. *Int J Radiat Oncol Biol Phys* 34:895-8. DOI: 0360301695020381 [pii].
- Smedby K.E., Baecklund E., Askling J. (2006) Malignant lymphomas in autoimmunity and inflammation: A review of risks, risk factors, and lymphoma characteristics. *Cancer Epidemiology Biomarkers & Prevention* 15:2069-2077. DOI: Doi 10.1158/1055-9965.Epi-06-0300.

- Smith J.S., Jenkins R.B. (2000) Genetic alterations in adult diffuse glioma: occurrence, significance, and prognostic implications. *Frontiers in Bioscience* 5:D213-31.
- Smith J.W. (2003) Cilengitide Merck. *Current Opinion in Investigational Drugs* 4:741-5.
- Sobel R.A., Ahmed A.S. (2001) White matter extracellular matrix chondroitin sulfate/dermatan sulfate proteoglycans in multiple sclerosis. *J Neuropathol Exp Neurol* 60:1198-207.
- Somasundaram K., Nijaguna M.B., Kumar D.M. (2009) Serum proteomics of glioma: methods and applications. *Expert Review of Molecular Diagnostics* 9:695-707. DOI: Doi 10.1586/Erm.09.52.
- Sommer, A., J. Hoffmann, R.B. Lichtner, M.R. Schneider, and K. Parczyk. 2003. Studies on the development of resistance to the pure antiestrogen Faslodex in three human breast cancer cell lines. *Journal of Steroid Biochemistry & Molecular Biology* 85:33-47.
- Songyang Z., Fanning A.S., Fu C., Xu J., Marfatia S.M., Chishti A.H., Crompton A., Chan A.C., Anderson J.M., Cantley L.C. (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* 275:73-77.
- Souhami L., Seiferheld W., Brachman D., Podgorsak E.B., Werner-Wasik M., Lustig R., Schultz C.J., Sause W., Okunieff P., Buckner J., Zamorano L., Mehta M.P., Curran W.J. (2004) Randomized comparison of stereotactic radiosurgery followed by conventional radiotherapy with carmustine to conventional radiotherapy with carmustine for patients with glioblastoma multiforme: Report of Radiation Therapy Oncology Group 93-05 protocol. *International Journal of Radiation Oncology Biology Physics* 60:853-860. DOI: DOI 10.1016/j.ijrobp.2004.04.011.
- Srivenugopal K.S., Yuan X.H., Friedman H.S., AliOsman F. (1996) Ubiquitination-dependent proteolysis of O-6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O-6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry* 35:1328-1334.
- Stallcup W.B., Beasley L. (1987) Bipotential Glial Precursor Cells of the Optic-Nerve Express the Ng2 Proteoglycan. *Journal of Neuroscience* 7:2737-2744.
- Stallcup W.B., Huang F.-J. (2008) A role for the NG2 proteoglycan in glioma progression. *Cell Adhesion & Migration* 2:192-201.
- Stanley B.A., Gundry R.L., Cotter R.J., Van Eyk J.E. (2004) Heart disease, clinical proteomics and mass spectrometry. *Disease Markers* 20:167-178.
- Stapels M.D., Barofsky D.F. (2004) Complementary use of MALDI and ESI for the HPLC-MS/MS analysis of DNA-binding proteins. *Analytical Chemistry* 76:5423-5430. DOI: Doi 10.1021/Ac030427z.
- Staugaitis S.M., Trapp B.D. (2009) NG2-positive glia in the human central nervous system. *Neuron Glia Biology* 5:35-44. DOI: Doi 10.1017/S1740925x09990342.

- Stegmuller J., Werner H., Nave K.A., Trotter J. (2003) The proteoglycan NG2 is complexed with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by the PDZ glutamate receptor interaction protein (GRIP) in glial progenitor cells - Implications for glial-neuronal signaling. *Journal of Biological Chemistry* 278:3590-3598. DOI: DOI 10.1074/jbc.M210010200.
- Stein G.H. (1979) T98g - Anchorage-Independent Human-Tumor Cell-Line That Exhibits Stationary Phase-G1 Arrest Invitro. *Journal of Cellular Physiology* 99:43-54.
- Stein M.E., Kuten A., Drumea K., Goldsher D., Tzuk-Shina Z. (1999) Carboplatin and etoposide for recurrent malignant glioma following surgical and radiotherapy failure: A clinical study conducted at the Northern Israel Oncology Center. *J Surg Oncol* 71:167-70. DOI: 10.1002/(SICI)1096-9098(199907)71:3<167::AID-JSO6>3.0.CO;2-V [pii].
- Stevens M.F.G., Hickman J.A., Langdon S.P., Chubb D., Vickers L., Stone R., Baig G., Goddard C., Gibson N.W., Slack J.A., Newton C., Lunt E., Fizames C., Lavelle F. (1987) Antitumor Imidazotetrazines .13. Antitumor-Activity and Pharmacokinetics in Mice of 8-Carbamoyl-3-Methyl-Imidazo[5,1-D]-1,2,3,5-Tetrazin-4(3h)-One (Crg 81045-M-and-B-39831), a Novel Drug with Potential as an Alternative to Dacarbazine. *Cancer Research* 47:5846-5852.
- Stewart D.J., Richard M.T., Hugenholtz H., Dennery J.M., Belanger R., Gerinlajoie J., Montpetit V., Nundy D., Prior J., Hopkins H.S. (1984) Penetration of Vp-16 (Etoposide) into Human Intracerebral and Extracerebral Tumors. *Journal of Neuro-Oncology* 2:133-139.
- Stewart L.A. (2002) Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet* 359:1011-8. DOI: S0140673602080911 [pii].
- Stiles C.D., Rowitch D.H. (2008) Glioma stem cells: A midterm exam. *Neuron* 58:832-846. DOI: DOI 10.1016/j.neuron.2008.05.031.
- Stockhausen M.T., Kristoffersen K., Poulsen H.S. (2010) The functional role of Notch signaling in human gliomas. *Neuro-Oncology* 12:199-211. DOI: DOI 10.1093/neuonc/nop022.
- Strik H.M., Hulper P., Erdlenbruch B., Meier J., Kowalewski A., Hemmerlein B., Gold R., Bahr M. (2006) Models of monocytic invasion into glioma cell aggregates. *Anticancer Research* 26:865-871.
- Strupat K., Rogniaux H., Van Dorsselaer A., Roth J., Vogl T. (2000) Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 are confirmed by electrospray ionization-mass analysis. *Journal of the American Society for Mass Spectrometry* 11:780-788.
- Stummer W., Pichlmeier U., Meinel T., Wiestler O.D., Zanella F., Hans-Jurgen R., Grp A.-G.S. (2006) Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncology* 7:392-401. DOI: Doi 10.1016/S1470-2045(06)70665-9.

- Stummer W., Stocker S., Novotny A., Heimann A., Sauer O., Kempfski O., Plesnila N., Wietzorrek J., Reulen H.J. (1998) In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. *Journal of Photochemistry and Photobiology B-Biology* 45:160-169.
- Stupp R., Weller M. (2010) 2010: neuro-oncology is moving! *Current Opinion in Neurology* 23:553-555. DOI: Doi 10.1097/Wco.0b013e3283407eed.
- Stupp R., Gander M., Leyvraz S., Newlands E. (2001) Current and future developments in the use of temozolomide for the treatment of brain tumours. *Lancet Oncology* 2:552-560.
- Stupp R., Kanner A., Engelhard H., Heidecke V., Taillibert S., Lieberman F.S., Dbalý V., Kirson E.D., Palti Y., Gutin P.H. (2010a) A prospective, randomized, open-label, phase III clinical trial of NovoTTF-100A versus best standard of care chemotherapy in patients with recurrent glioblastoma [abstract]. *J Clin Oncol* LBA2007.
- Stupp R., Bent M.J.V.D., Erridge S.C., Reardon D.A., Hong Y., Wheeler H., Hegi M., Perry J.R., Picard M., Weller M. (2010b) Cilengitide in newly diagnosed glioblastoma with MGMT promoter methylation: Protocol of a multicenter, randomized, open-label, controlled phase III trial (CENTRIC) [suppl; abstract TPS152]. *J Clin Oncol* 28.
- Stupp R., Hegi M.E., Neyns B., Goldbrunner R., Schlegel U., Clement P.M.J., Grabenbauer G.G., Ochsenbein A.F., Simon M., Dietrich P.-Y., Pietsch T., Hicking C., Tonn J.-C., Diserens A.-C., Pica A., Hermisson M., Krueger S., Picard M., Weller M. (2010c) Phase I/IIa study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. *Journal of Clinical Oncology* 28:2712-8.
- Stupp R., Mason W.P., van den Bent M.J., Weller M., Fisher B., Taphoorn M.J., Belanger K., Brandes A.A., Marosi C., Bogdahn U., Curschmann J., Janzer R.C., Ludwin S.K., Gorlia T., Allgeier A., Lacombe D., Cairncross J.G., Eisenhauer E., Mirimanoff R.O., European Organisation for R., Treatment of Cancer Brain T., Radiotherapy G., National Cancer Institute of Canada Clinical Trials G. (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New England Journal of Medicine* 352:987-996.
- Stupp R., Hegi M.E., Mason W.P., van den Bent M.J., Taphoorn M.J.B., Janzer R.C., Ludwin S.K., Allgeier A., Fisher B., Belanger K., Hau P., Brandes A.A., Gijtenbeek J., Marosi C., Vecht C.J., Mokhtari K., Wesseling P., Villa S., Eisenhauer E., Gorlia T., Weller M., Lacombe D., Cairncross J.G., Mirimanoff R.O., Treatment E.O.R., Grp C.B.T., Grp R.O., Trials N.C.I.C.C. (2009) Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncology* 10:459-466. DOI: Doi 10.1016/S1470-2045(09)70025-7.

- Sturgeon C.M., Duffy M.J., Stenman U.H., Lilja H., Brunner N., Chan D.W., Babaian R., Bast R.C., Dowell B., Esteva F.J., Haglund C., Harbeck N., Hayes D.F., Holten-Andersen M., Klee G.G., Lamerz R., Looijenga L.H., Molina R., Nielsen H.J., Rittenhouse H., Semjonow A., Shih I.M., Sibley P., Soletormos G., Stephan C., Sokoll L., Hoffman B.R., Diamandis E.P. (2008) National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for Use of Tumor Markers in Testicular, Prostate, Colorectal, Breast, and Ovarian Cancers. *Clinical Chemistry* 54:E11-E79. DOI: DOI 10.1373/clinchem.2008.105601.
- Sugiarto S., Persson A., Munoz E.G., Waldhuber M., Vandenberg S., Stallcup W., Philips J., Berger M.S., Bergers G., Weiss W.A., Petritsch C. (2011) Asymmetry-Defective Oligodendrocyte Progenitors Are Glioma Precursors. *Neuro-Oncology* 13:25-25.
- Sung P.A., Libura J., Richardson C. (2006) Etoposide and illegitimate DNA double-strand break repair in the generation of MLL translocations: New insights and new questions. *DNA Repair* 5:1109-1118. DOI: 10.1016/j.dnarep.2006.05.018.
- Svendsen A., Verhoeff J.J.C., Immervoll H., Brogger J.C., Kmiecik J., Poli A., Netland I.A., Prestegarden L., Planaguma J., Torsvik A., Kjersem A.B., Sakariassen P.O., Heggdal J.I., Van Furth W.R., Bjerkvig R., Lund-Johansen M., Enger P.O., Felsberg J., Brons N.H.C., Tronstad K.J., Waha A., Chekenya M. (2011) Expression of the progenitor marker NG2/CSPG4 predicts poor survival and resistance to ionising radiation in glioblastoma. *Acta Neuropathologica* 122:495-510. DOI: DOI 10.1007/s00401-011-0867-2.
- Swope M., Sun H.W., Blake P.R., Lolis E. (1998) Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor. *Embo Journal* 17:3534-3541.
- Syeda F., Ahsan F., Nunez D.A. (2007) Quality of life outcome analysis in patients undergoing submandibular duct repositioning surgery for sialorrhoea. *Journal of Laryngology and Otology* 121:555-558. DOI: Doi 10.1017/S0022215106004105.
- Takahashi H., Shibuya M. (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clinical Science* 109:227-241. DOI: Doi 10.1042/Cs20040370.
- Takano S., Yoshitomi H., Togawa A., Sogawa K., Shida T., Kimura F., Shimizu H., Tomonaga T., Nomura F., Miyazaki M. (2008) Apolipoprotein C-1 maintains cell survival by preventing from apoptosis in pancreatic cancer cells. *Oncogene* 27:2810-2822. DOI: DOI 10.1038/sj.onc.1210951.
- Tanaka K., Waki H., Ido Y., Akita S., Yoshida Y., Yoshida T., Matsuo T. (1988) Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 2:151-153. DOI: 10.1002/rcm.1290020802.
- Tang K., Jin Q., Yan W., Zhang W., You G., Liu Y., Jiang T. (2011) Clinical correlation of MGMT protein expression and promoter methylation in Chinese glioblastoma patients. *Med Oncol*. DOI: 10.1007/s12032-011-9901-4.

- Tentori L., Graziani G. (2002) Pharmacological strategies to increase the antitumor activity of methylating agents. *Current Medicinal Chemistry* 9:1285-1301.
- Theys J., Jutten B., Dubois L., Rouschop K.M.A., Chiu R.K., Li Y.N., Paesmans K., Lambin P., Lammering G., Wouters B.G. (2009) The deletion mutant EGFRvIII significantly contributes to stress resistance typical for the tumour microenvironment. *Radiotherapy and Oncology* 92:399-404. DOI: DOI 10.1016/j.radonc.2009.06.017.
- Thibault D.B., Gillam C.J., Grey A.C., Han J., Schey K.L. (2008) MALDI tissue profiling of integral membrane proteins from ocular tissues. *Journal of the American Society for Mass Spectrometry* 19:814-822. DOI: DOI 10.1016/j.jasms.2008.03.002.
- Thorat S., Daly T.M., Bergman L.W., Burns J.M. (2010) Elevated Levels of the Plasmodium yoelii Homologue of Macrophage Migration Inhibitory Factor Attenuate Blood-Stage Malaria. *Infection and Immunity* 78:5151-5162. DOI: Doi 10.1128/iai.00277-10.
- Tillet E., Ruggiero F., Nishiyama A., Stallcup W.B. (1997) The membrane-spanning proteoglycan NG2 binds to collagens V and VI through the central nonglobular domain of its core protein. *Journal of Biological Chemistry* 272:10769-10776.
- Tillet E., Gential B., Garrone R., Stallcup W.B. (2002) NG2 proteoglycan mediates beta1 integrin-independent cell adhesion and spreading on collagen VI. *J Cell Biochem* 86:726-36. DOI: 10.1002/jcb.10268.
- Toledo F., Wahl G.M. (2006) Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* 6:909-23. DOI: nrc2012 [pii] 10.1038/nrc2012.
- Tonn J.C., Stummer W. (2008) Fluorescence-guided resection of malignant gliomas using 5-aminolevulinic acid: practical use, risks, and pitfalls. *Clin Neurosurg* 55:20-6.
- Tortosa A., Vinolas N., Villa S., Verger E., Gil J.M., Brell M., Caral L., Pujol T., Acebes J.J., Ribalta T., Ferrer I., Graus F. (2003) Prognostic implication of clinical, radiologic, and pathologic features in patients with anaplastic gliomas. *Cancer* 97:1063-71.
- Traub F., Feist H., Kreipe H.H., Pich A. (2005) SELDI-MS-based expression profiling of ductal invasive and lobular invasive human breast carcinomas. *Pathol Res Pract* 201:763-70. DOI: S0344-0338(05)00167-6 [pii] 10.1016/j.prp.2005.08.011.
- Tricot G.J. (2002) New insights into role of microenvironment in multiple myeloma. *International Journal of Hematology* 76:334-336.
- Trotter J., Karram K., Nishiyama A. (2010) NG2 cells: Properties, progeny and origin. *Brain Research Reviews* 63:72-82. DOI: DOI 10.1016/j.brainresrev.2009.12.006.
- Tyers M., Mann M. (2003) From genomics to proteomics. *Nature* 422:193-197. DOI: Doi 10.1038/Nature01510.
- Uchida T., Fukawa A., Uchida M., Fujita K., Saito K. (2002) Application of a novel protein biochip technology for detection and identification of rheumatoid arthritis biomarkers in synovial fluid. *Journal of Proteome Research* 1:495-499. DOI: Doi 10.1021/Pr025531w.

- Ueda E.K.M., Gout P.W., Morganti L. (2003) Current and prospective applications of metal ion-protein binding. *Journal of Chromatography A* 988:1-23. DOI: Doi 10.1016/S0021-9673(02)02057-5.
- Uggla B., Tina E., Nahi H., Paul C., Hoglund M., Sirsjo A., Tidefelt U. (2007) Topoisomerase II alpha mRNA and protein expression vs. in vitro drug resistance and clinical outcome in acute leukaemia. *International Journal of Oncology* 31:153-160.
- Valore E.V., Ganz T. (1992) Posttranslational Processing of Defensins in Immature Human Myeloid Cells. *Blood* 79:1538-1544.
- van den Bent M.J., Gravendeel L.A., Gorlia T., Kros J.M., Lapre L., Wesseling P., Teepen J.L., Idbaih A., Sanson M., Smitt P.A.E.S., French P.J. (2011) A Hypermethylated Phenotype Is a Better Predictor of Survival than MGMT Methylation in Anaplastic Oligodendroglial Brain Tumors: A Report from EORTC Study 26951. *Clinical Cancer Research* 17:7148-7155. DOI: Doi 10.1158/1078-0432.Ccr-11-1274.
- van den Bent M.J., Dubbink H.J., van der Lee-Haarloo C.R., Dinjens W.N.M., Hegi M., Jeuken J.W.M., Sanson M., Idbaih A., Brandes A.A., Lacombe D., Gorlia T., Kros J.M. (2009a) Mgmt Promoter Gene Methylation as Determined with Multiplex Ligation-Dependent Probe Amplification (Mlpa) Is Prognostic for Survival after Radiotherapy but Not Predictive for Outcome to Chemotherapy in Oligodendroglial Tumors. *Neuro-Oncology* 11:904-905.
- van den Bent M.J., Brandes A.A., Rampling R., Kouwenhoven M.C.M., Kros J.M., Carpentier A.F., Clement P.M., Frenay M., Campone M., Baurain J.F., Armand J.P., Taphoorn M.J.B., Tosoni A., Kletzl H., Klughammer B., Lacombe D., Gorlia T. (2009b) Randomized Phase II Trial of Erlotinib Versus Temozolomide or Carmustine in Recurrent Glioblastoma: EORTC Brain Tumor Group Study 26034. *Journal of Clinical Oncology* 27:1268-1274. DOI: Doi 10.1200/Jco.2008.17.5984.
- van Nifterik K.A., van den Berg J., van der Meide W.F., Ameziane N., Wedekind L.E., Steenbergen R.D.M., Leenstra S., Lafleur M.V.M., Slotman B.J., Stalpers L.J.A., Sminia P. (2010) Absence of the MGMT protein as well as methylation of the MGMT promoter predict the sensitivity for temozolomide. *British Journal of Cancer* 103:29-35. DOI: DOI 10.1038/sj.bjc.6605712.
- van Wetering S., Mannesse-Lazeroms S.P.G., van Sterkenburg M.A.J.A., Hiemstra P.S. (2002) Neutrophil defensins stimulate the release of cytokines by airway epithelial cells: modulation by dexamethasone. *Inflammation Research* 51:8-15.
- van Winden A.W.J., Gast M.C.W., Beijnen J.H., Rutgers E.J.T., Grobbee D.E., Peeters P.H.M., van Gils C.H. (2009) Validation of previously identified serum biomarkers for breast cancer with SELDI-TOF MS: a case control study. *Bmc Medical Genomics* 2. DOI: Artn 4 Doi 10.1186/1755-8794-2-4.
- Vassetzky Y.S., Alghisi G.C., Gasser S.M. (1995) DNA topoisomerase II mutations and resistance to anti-tumor drugs. *Bioessays* 17:767-74. DOI: 10.1002/bies.950170906.

- Veeravagu A., Liu Z.F., Niu G., Chen K., Jia B., Cai W.B., Jin C.J., Hsu A.R., Connolly A.J., Tse V., Wang F., Chen X.Y. (2008) Integrin $\alpha(v)\beta(3)$ -Targeted Radioimmunotherapy of Glioblastoma Multiforme. *Clinical Cancer Research* 14:7330-7339. DOI: Doi 10.1158/1078-0432.Ccr-08-0797.
- Verhaak R.G.W., Hoadley K.A., Purdom E., Wang V., Qi Y., Wilkerson M.D., Miller C.R., Ding L., Golub T., Mesirov J.P., Alexe G., Lawrence M., O'Kelly M., Tamayo P., Weir B.A., Gabriel S., Winckler W., Gupta S., Jakkula L., Feiler H.S., Hodgson J.G., James C.D., Sarkaria J.N., Brennan C., Kahn A., Spellman P.T., Wilson R.K., Speed T.P., Gray J.W., Meyerson M., Getz G., Perou C.M., Hayes D.N. (2010) Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma Characterized by Abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17:98-110. DOI: DOI: 10.1016/j.ccr.2009.12.020.
- Virsik-Kopp P., Rave-Frank M., Hofman-Huther H., Schmidberger H. (2003) Role of DNA-PK in the process of aberration formation as studied in irradiated human glioblastoma cell lines M059K and M059J. *International Journal of Radiation Biology* 79:61-68. DOI: 10.1080/0955300021000038644.
- Vitaz T.W., Warnke P.C., Tabar V., Gutin P.H. (2005) Brachytherapy for brain tumors. *Journal of Neuro-Oncology* 73:71-86. DOI: DOI 10.1007/s11060-004-2352-4.
- Vlahou A., Laronga C., Wilson L., Gregory B., Fournier K., McGaughey D., Perry R.R., Wright G.L., Jr., Semmes O.J. (2003) A novel approach toward development of a rapid blood test for breast cancer. *Clin Breast Cancer* 4:203-9.
- Vogl T., Roth J., Sorg C., Hillenkamp F., Strupat K. (1999) Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption/ionization mass spectrometry. *Journal of the American Society for Mass Spectrometry* 10:1124-1130.
- Vorderwulbecke S., Cleverley S., Weinberger S., Wiesner A. (2005) Protein quantification by the SELDI-TOF-MS-based protein Chip (R) system. *Molecular & Cellular Proteomics* 4:S381-S381.
- Vredenburgh J.J., Desjardins A., Herndon J.E., Dowel J.M., Reardon D.A., Quinn J.A., Rich J.N., Sathornsumetee S., Gururangan S., Wagner M., Bigner D.D., Friedman A.H., Friedman H.S. (2007) Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. *Clinical Cancer Research* 13:1253-1259. DOI: Doi 10.1158/1078-0432.Ccr-06-2309.
- Wadsworth J.T., Somers K.D., Stack B.C., Jr., Cazares L., Malik G., Adam B.L., Wright G.L., Jr., Semmes O.J., Wadsworth J.T., Somers K.D., Stack B.C., Jr., Cazares L., Malik G., Adam B.-L., Wright G.L., Jr., Semmes O.J. (2004) Identification of patients with head and neck cancer using serum protein profiles. *Archives of Otolaryngology -- Head & Neck Surgery* 130:98-104.

- Wang J., Svendsen A., Kmiecik J., Immervol H., Skaftnesmo K.O., Planaguma J., Reed R.K., Bjerkvig R., Miletic H., Enger P.O., Rygh C.B., Chekenya M. (2011a) Targeting the NG2/CSPG4 Proteoglycan Retards Tumour Growth and Angiogenesis in Preclinical Models of GBM and Melanoma. *Plos One* 6. DOI: ARTN e23062 DOI 10.1371/journal.pone.0023062.
- Wang J.C. (1971) Interaction between DNA and an Escherichia coli protein omega. *J Mol Biol* 55:523-33.
- Wang J.C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Reviews Molecular Cell Biology* 3:430-40.
- Wang Q., Beck W.T. (1998) Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53. *Cancer Research* 58:5762-9.
- Wang Q.J., Zambetti G.P., Suttle D.P. (1997a) Inhibition of DNA topoisomerase II alpha gene expression by the p53 tumor suppressor. *Molecular and Cellular Biology* 17:389-397.
- Wang S.I., Puc J., Li J., Bruce J.N., Cairns P., Sidransky D., Parsons R. (1997b) Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Research* 57:4183-4186.
- Wang X.B., Tian X.Y., Li Y., Li B., Li Z. (2011b) Elevated expression of macrophage migration inhibitory factor correlates with tumor recurrence and poor prognosis of patients with gliomas. *J Neurooncol.* DOI: 10.1007/s11060-011-0640-3.
- Wang X.W., Harris C.C. (1997) p53 tumor-suppressor gene: Clues to molecular carcinogenesis. *Journal of Cellular Physiology* 173:247-255.
- Wasinger V.C., Cordwell S.J., Cerpapojak A., Yan J.X., Gooley A.A., Wilkins M.R., Duncan M.W., Harris R., Williams K.L., Humpherysmith I. (1995) Progress with Gene-Product Mapping of the Mollicutes - Mycoplasma-Genitalium. *Electrophoresis* 16:1090-1094.
- Watanabe K., Kanaya H., Fujiyama Y., Kim P. (2002) Combination chemotherapy using carboplatin (JM-8) and etoposide (JET therapy) for recurrent malignant gliomas: A phase II study. *Acta Neurochirurgica* 144:1265-1270. DOI: 10.1007/s00701-002-1023-5.
- Weis S.M., Cheres D.A. (2011) Integrins in Angiogenesis and Cancer. *Cold Spring Harb Perspect Med* 1:a006478. DOI: 10.1101/cshperspect.a006478 a006478 [pii].
- Weller M. (2011) Novel diagnostic and therapeutic approaches to malignant glioma. *Swiss Medical Weekly* 141. DOI: ARTN w13210 DOI 10.4414/smw.2011.13210.
- Weller M., Stupp R., Reifenberger G., Brandes A.A., van den Bent M.J., Wick W., Hegi M.E. (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nature Reviews Neurology* 6:39-51. DOI: DOI 10.1038/nrneurol.2009.197.

- Weller M., Berger H., Hartmann C., Schramm J., Westphal M., Simon M., Goldbrunner R., Krex D., Steinbach J.P., Ostertag C.B., Loeffler M., Pietsch T., von Deimlin A., Network G.G. (2007) Combined 1p/19q loss in oligodendroglial tumors: Predictive or prognostic biomarker? *Clinical Cancer Research* 13:6933-6937. DOI: Doi 10.1158/1078-0432.Ccr-07-0573.
- Wen P.Y., Kesari S. (2008) Malignant gliomas in adults. *New England Journal of Medicine* 359:492-507.
- Wen P.Y., Yung W.K.A., Lamborn K.R., Dahia P.L., Wang Y.F., Peng B., Abrey L.E., Raizer J., Cloughesy T.F., Fink K., Gilbert M., Chang S., Junck L., Schiff D., Lieberman F., Fine H.A., Mehta M., Robins H.I., DeAngelis L.M., Groves M.D., Puduvalli V.K., Levin V., Conrad C., Maher E.A., Aldape K., Hayes M., Letvak L., Egorin M.J., Capdeville R., Kaplan R., Murgo A.J., Stiles C., Prados M.D. (2006a) Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. *Clinical Cancer Research* 12:4899-4907. DOI: Doi 10.1158/1078-0432.Ccr-06-0773.
- Wen Y.F., Makagiansar I.T., Fukushi J., Liu F.T., Fukuda M.N., Stallcup W.B. (2006b) Molecular basis of interaction between NG2 proteoglycan and galectin-3. *Journal of Cellular Biochemistry* 98:115-127. DOI: Doi 10.1002/Jcb.20768.
- Werndle M.C., Jones T.L., Papadopoulos M.C. (2011) Neurosurgical Management of Gliomas, Diagnostic Techniques and Surgical Management of Brain Tumors. Ana Lucia Abujamra (Ed.), InTech SBN: 978-953-307-589-1.
- Wesseling P., Schlingemann R.O., Rietveld F.J.R., Link M., Burger P.C., Ruiter D.J. (1995) Early and Extensive Contribution of Pericytes/Vascular Smooth-Muscle Cells to Microvascular Proliferation in Glioblastoma-Multiforme - an Immuno-Light and Immunoelectron Microscopic Study. *Journal of Neuropathology and Experimental Neurology* 54:304-310.
- Wibom C., Pettersson F., Sjostrom M., Henriksson R., Johansson M., Bergenheim A.T. (2006) Protein expression in experimental malignant glioma varies over time and is altered by radiotherapy treatment. *British Journal of Cancer* 94:1853-1863. DOI: DOI 10.1038/sj.bjc.6603190.
- Wick W., Puduvalli V.K., Chamberlain M.C., van den Bent M.J., Carpentier A.F., Cher L.M., Mason W., Weller M., Hong S., Musib L., Liepa A.M., Thornton D.E., Fine H.A. (2010) Phase III Study of Enzastaurin Compared With Lomustine in the Treatment of Recurrent Intracranial Glioblastoma. *Journal of Clinical Oncology* 28:1168-1174. DOI: Doi 10.1200/Jco.2009.23.2595.
- Wick W., Hartmann C., Engel C., Stoffels M., Felsberg J., Stockhammer F., Sabel M.C., Koeppen S., Ketter R., Meyermann R., Rapp M., Meisner C., Kortmann R.D., Pietsch T., Wiestler O.D., Ernemann U., Bamberg M., Reifenberger G., von Deimling A., Weller M. (2009) NOA-04 Randomized Phase III Trial of Sequential Radiochemotherapy of Anaplastic Glioma With Procarbazine, Lomustine, and Vincristine or Temozolomide. *Journal of Clinical Oncology* 27:5874-5880. DOI: Doi 10.1200/Jco.2009.23.6497.

- Willems P.W.A., Taphoorn M.J.B., Burger H., van der Sprenkel J.W.B., Tulleken C.A.F. (2006) Effectiveness of neuronavigation in resecting solitary intracerebral contrast-enhancing tumors: a randomized controlled trial. *Journal of Neurosurgery* 104:360-368.
- Williams S.A., Slavin D.E., Wagner J.A., Webster C.J. (2006) Opinion - A cost-effectiveness approach to the qualification and acceptance of biomarkers. *Nature Reviews Drug Discovery* 5:897-902. DOI: Doi 10.1038/Nrd2174.
- Wilson L.L., Tran L., Morton D.L., Hoon D.S., Wilson L.L., Tran L., Morton D.L., Hoon D.S.B. (2004) Detection of differentially expressed proteins in early-stage melanoma patients using SELDI-TOF mass spectrometry. *Annals of the New York Academy of Sciences* 1022:317-22.
- Wilstermann A.M., Osheroff N. (2003) Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. *Current Topics in Medicinal Chemistry* 3:321-338.
- Wilstermann A.M., Bender R.P., Godfrey M., Choi S., Anklin C., Berkowitz D.B., Osheroff N., Graves D.E., Wilstermann A.M., Bender R.P., Godfrey M., Choi S., Anklin C., Berkowitz D.B., Osheroff N., Graves D.E. (2007) Topoisomerase II - drug interaction domains: identification of substituents on etoposide that interact with the enzyme. *Biochemistry* 46:8217-25.
- Winner M., Koong A.C., Rendon B.E., Zundel W., Mitchell R.A. (2007) Amplification of tumor hypoxic responses by macrophage migration inhibitory factor-dependent hypoxia-inducible factor stabilization. *Cancer Research* 67:186-193. DOI: Doi 10.1158/0008-5472.Can-06-3292.
- Witz I.P., Levy-Nissenbaum O. (2006) The tumor microenvironment in the post-PAGET era. *Cancer Letters* 242:1-10.
- Woessner R.D., Mattern M.R., Mirabelli C.K., Johnson R.K., Drake F.H. (1991) Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ* 2:209-14.
- Wolburg H., Lippoldt A. (2002) Tight junctions of the blood-brain barrier: Development, composition and regulation. *Vascular Pharmacology* 38:323-337.
- Wrensch M., Minn Y., Chew T., Bondy M., Berger M.S. (2002) Epidemiology of primary brain tumors: current concepts and review of the literature. *Neuro-Oncology* 4:278-99.
- Wright G.L., Jr. (2002) SELDI proteinchip MS: a platform for biomarker discovery and cancer diagnosis. *Expert Rev Mol Diagn* 2:549-63. DOI: ERM020605 [pii].
- Wu W.W., Wang G.H., Baek S.J., Shen R.F. (2006) Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *Journal of Proteome Research* 5:651-658. DOI: Doi 10.1021/Pr050405o.
- Xiao H., Mao Y., Desai S.D., Zhou N., Ting C.Y., Hwang J., Liu L.F., Xiao H., Mao Y., Desai S.D., Zhou N., Ting C.-Y., Hwang J., Liu L.F. (2003) The topoisomerase IIbeta circular clamp arrests transcription and signals a 26S proteasome pathway. *Proceedings of the National Academy of Sciences of the United States of America* 100:3239-44.

- Xu G., Xiang C.Q., Lu Y., Kang X.N., Liao P., Ding Q., Zhang Y.F., Xu G., Xiang C.Q., Lu Y., Kang X.N., Liao P., Ding Q., Zhang Y.F. (2009) Application of SELDI-TOF-MS to identify serum biomarkers for renal cell carcinoma. *Cancer Letters* 282:205-13.
- Xu W., Yang H., Liu Y., Yang Y., Wang P., Kim S.H., Ito S., Yang C., Xiao M.T., Liu L.X., Jiang W.Q., Liu J., Zhang J.Y., Wang B., Frye S., Zhang Y., Xu Y.H., Lei Q.Y., Guan K.L., Zhao S.M., Xiong Y. (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* 19:17-30. DOI: S1535-6108(10)00527-1 [pii] 10.1016/j.ccr.2010.12.014.
- Xu X., Zhao J.Y., Xu Z., Peng B.Z., Huang Q.H., Arnold E., Ding J.P. (2004) Structures of human cytosolic NADP-dependent isocitrate dehydrogenase reveal a novel self-regulatory mechanism of activity. *Journal of Biological Chemistry* 279:33946-33957. DOI: DOI 10.1074/jbc.M404298200.
- Xue A., Scarlett C.J., Chung L., Butturini G., Scarpa A., Gandy R., Wilson S.R., Baxter R.C., Smith R.C. (2010) Discovery of serum biomarkers for pancreatic adenocarcinoma using proteomic analysis. *British Journal of Cancer* 103:391-400. DOI: DOI 10.1038/sj.bjc.6605764.
- Yamaguchi N., Isomoto H., Mukae H., Ishimoto H., Ohnita K., Shikuwa S., Mizuta Y., Nakazato M., Kohno S. (2009) Concentrations of alpha- and beta-defensins in plasma of patients with inflammatory bowel disease. *Inflammation Research* 58:192-197. DOI: DOI 10.1007/s00011-008-8120-8.
- Yamamoto T., Nakai K., Matsumura A. (2008) Boron neutron capture therapy for glioblastoma. *Cancer Letters* 262:143-152. DOI: DOI 10.1016/j.canlet.2008.01.021.
- Yan H., Parsons D.W., Jin G.L., McLendon R., Rasheed B.A., Yuan W.S., Kos I., Batnig-Haberle I., Jones S., Riggins G.J., Friedman H., Friedman A., Reardon D., Herndon J., Kinzler K.W., Velculescu V.E., Vogelstein B., Bigner D.D. (2009) IDH1 and IDH2 Mutations in Gliomas. *New England Journal of Medicine* 360:765-773.
- Yang J.B., Price M.A., Neudauer C.L., Wilson C., Ferrone S., Xia H., Iida J., Simpson M.A., McCarthy J.B. (2004a) Melanoma chondroitin sulfate proteoglycan enhances FAK and ERK activation by distinct mechanisms. *Journal of Cell Biology* 165:881-891. DOI: DOI 10.1083/jcb.200403174.
- Yang Y.L., Li C.C.H., Weissman A.M. (2004b) Regulating the p53 system through ubiquitination. *Oncogene* 23:2096-2106. DOI: 10.1038/sj.onc.1207411.
- Yates A.J. (1992) An overview of principles for classifying brain tumors. *Molecular & Chemical Neuropathology* 17:103-20.
- Yoshino A., Ogino A., Yachi K., Ohta T., Fukushima T., Watanabe T., Katayama Y., Okamoto Y., Naruse N., Sano E., Tsumoto K. (2010) Gene expression profiling predicts response to temozolomide in malignant gliomas. *International Journal of Oncology* 36:1367-1377. DOI: Doi 10.3892/Ijo_00000621.

- Yu J.K., Zheng S., Tang Y., Li L. (2005) An integrated approach utilizing proteomics and bioinformatics to detect ovarian cancer. *J Zhejiang Univ Sci B* 6:227-31. DOI: 10.1631/jzus.2005.B0227.
- Yuan X.P., Curtin J., Xiong Y.Z., Liu G.T., Waschmann-Hogiu S., Farkas D.L., Black K.L., Yu J.S. (2004) Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 23:9392-9400. DOI: DOI 10.1038/sj.onc.1208311.
- Yung W.K.A., Prados M.D., Yaya-Tur R., Rosenfeld S.S., Brada M., Friedman H.S., Albright R., Olson J., Chang S.M., O'Neill A.M., Friedman A.H., Bruner J., Yue N., Dugan M., Zaknoen S., Levin V.A., Grp T.B.T. (1999) Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapse. *Journal of Clinical Oncology* 17:2762-2771.
- Zechiedrich E.L., Christiansen K., Andersen A.H., Westergaard O., Osheroff N. (1989) Double-Stranded DNA Cleavage Religation Reaction of Eukaryotic Topoisomerase-II - Evidence for a Nicked DNA Intermediate. *Biochemistry* 28:6229-6236.
- Zhang H., Wu G., Tu H., Huang F. (2007a) Discovery of serum biomarkers in astrocytoma by SELDI-TOF MS and proteinchip technology. *J Neurooncol* 84:315-23. DOI: 10.1007/s11060-007-9376-5.
- Zhang H., Kong B.H., Qu X., Jia L., Deng B.P., Yang Q.F. (2006) Biomarker discovery for ovarian cancer using SELDI-TOF-MS. *Gynecologic Oncology* 102:61-66. DOI: DOI 10.1016/j.ygyno.2005.11.029.
- Zhang H., Wu G., Tu H., Huang F., Zhang H., Wu G., Tu H., Huang F. (2007b) Discovery of serum biomarkers in astrocytoma by SELDI-TOF MS and proteinchip technology. *Journal of Neuro-Oncology* 84:315-23.
- Zhang J.H., Stevens M.F.G., Laughton C.A., Madhusudan S., Bradshaw T.D. (2010) Acquired Resistance to Temozolomide in Glioma Cell Lines: Molecular Mechanisms and Potential Translational Applications. *Oncology* 78:103-114. DOI: Doi 10.1159/000306139.
- Zhang X.P., Zheng G., Zou L., Liu H.L., Hou L.H., Zhou P., Yin D.D., Zheng Q.J., Liang L., Zhang S.Z., Feng L., Yao L.B., Yang A.G., Han H., Chen J.Y. (2008) Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. *Molecular and Cellular Biochemistry* 307:101-108. DOI: DOI 10.1007/s11010-007-9589-0.
- Zhang Z., Bast R.C., Yu Y.H., Li J.N., Sokoll L.J., Rai A.J., Rosenzweig J.M., Cameron B., Wang Y.Y., Meng X.Y., Berchuck A., van Haaften-Day C., Hacker N.F., de Bruijn H.W.A., van der Zee A.G.J., Jacobs I.J., Fung E.T., Chan D.W. (2004) Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Research* 64:5882-5890.
- Zhou Q., Wang S., Anderson D.J. (2000) Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* 25:331-43. DOI: S0896-6273(00)80898-3 [pii].

- Zhou X.P., Li Y.J., Hoang-Xuan K., Laurent-Puig P., Mokhtari K., Longy M., Sanson M., Delattre J.Y., Thomas G., Hamelin R. (1999) Mutational analysis of the PTEN gene in gliomas: Molecular and pathological correlations. *International Journal of Cancer* 84:150-154.
- Zhou Z., Zwelling L.A., Ganapathi R., Kleinerman E.S. (2001) Enhanced etoposide sensitivity following adenovirus-mediated human topoisomerase II alpha gene transfer is independent of topoisomerase II beta. *British Journal of Cancer* 85:747-751. DOI: 10.1054/bjoc.2001.1966.
- Zimmermann D.R., Dours-Zimmermann M.T. (2008) Extracellular matrix of the central nervous system: from neglect to challenge. *Histochemistry and Cell Biology* 130:635-653. DOI: DOI 10.1007/s00418-008-0485-9.
- Zini N., Martelli A.M., Sabatelli P., Santi S., Negri C., Ricotti G.C.B.A., Maraldi N.M. (1992) The 180-Kda Isoform of Topoisomerase-II Is Localized in the Nucleolus and Belongs to the Structural Elements of the Nucleolar Remnant. *Experimental Cell Research* 200:460-466.