ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATING THE EFFECT OF ALTERED BAG1 GENE EXPRESSION IN HUMAN MCF7 BREAST CANCER CELL LINE

M.Sc. THESIS

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Thesis Advisor: Assist. Prof. Dr. Gizem DİNLER DOĞANAY

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İNSAN MCF7 MEME KANSERİ HÜCRE HATTINDA BAG1 GEN İFADESİNDEKİ DEĞİŞİMİN ETKİLERİNİN ARAŞTIRILMASI

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To my Best Parents (mama & baba) & also my Best Advisor

Imagine there's NO Cancer
It's easy if we try, No pain or suffering
or waiting just to Die….

FOREWORD

I would like to thank to my advisor Assoc. Prof. Dr. Gizem DİNLER - DOĞANAY for her motivations and supports throughout my master's study. This project would not exist without her unlimited support and inspiration. I want to send my endless gratitudes to all the teachers who prepared me to these days. And to my Best family who supported me all the time even when I was in Turky.

May 2013 Sevil BEHNOOD

MolecularBiologyGeneticbiotechnolog

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ABBREVIATIONS

µg : Microgram
µL : Microliter
µm : Micrometer
µM : Micromolar
Bad : Bcl-2-associated death promoter
Bag-1 : Bcl-2-associated athanogene
Bax : Bcl-2 homologous antagonist/killer
Bcl-2 : B-cell lymphoma-2
bp : Base pair
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
E.coli : Escherichia coli
FBS : Fetal bovine berum
g : Gram
Kb : Kilo base
kDa : Kilo dalton
L : Liter
LB : Luria-Bertani Broth
M : Molar
MAP : Mitogen-activated protein
MAPK : Mitogen-activated protein kinase
mg : Miligram
min : Minute
mL : Mililiter
mm : Milimeter
mM : Milimolar
mRNA : Messenger ribonucleic acid
NCBI : National Center for Biotechnology Information
ng : Nanogram
nM : Nanomolar
OD : Optical Density
PBS : Phosphate Buffered Saline
qPCR : Real time Polymerase chain reaction
PI : Propidium Iodide
pmol : picomole
RNA : Ribonucleic acid
rpm : Revolutions per minute
siRNA : Small interfering RNA
SOC : Super Optimal Broth with catabolite repression
TAP : Tandem Affinity Purification

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INVESTIGATING THE EFFECT OF ALTERED BAG1 GENE EXPRESSION IN HUMAN MCF7 BREAST CANCER CELL LINE

SUMMARY

Bcl-2 associated athanogene family (athano = against death in Greek) proteins are discovered by co-immunoprecipitation with Bcl-2 anti-apoptotic protein. Bag genes are evolutionally conserved and their homologues exist in yeasts, plants and animals. Bag family members control the cell homeostais in two different ways: (1) They contribute to maintain cell's vital activity under normal and stress conditions via regulating both positively and negatively the cycle of Hsp70/Hsc70 molecular chaperone system. As a specific example, the direction of the cell balance under normal and stress conditions are maintained by competitive binding of Hsp70 or Raf-1 to Bag-1. (2) They form complexes with transcription factors and nuclear hormone receptors which are controlling various physiological processes such as apoptosis, tumorigenesis, neural differentiation and cell cycle independent from Hsp70/Hsc70 system. Expression levels and intracellular localization of Bag-1 family members differ in human in some complex diseases such as AIDS and Parkinson Disease in comparison with normal cells. For instance, the studies on breast cancer cells showed that Bag-1 expression can be used as a prognostic marker to monitor the disease progression. Recently, it is postulated that high Bag-1 expression is also a prognostic marker for colon cancer. Bag-1 isoforms appear to be differently localized in the cells. Main Bag-1 isoforms which are observed in humans are Bag-1S (36 kDa), Bag-1M (46 kDa) and Bag-1L (50 kDa). There is also another isoform of Bag-1, 29 kDa, however, since it is very scarce in the cell and thus is not consistently detected. Bag-1 isoforms are produced by alternative translation initiation of single mRNA transcript; thereby they differ in their N-terminus related to translation initiation. All of Bag-1 isoforms commonly include ubiquitin-like domain (ULD) and Bag domain (BD) in their C-terminus. Even though ULD domain function is not known clearly its evolutional conservation and relations with proteosom machinary indicates that ULD is necessary for stress tolerance. Bag-1L is localized in nucleus by means of its nuclear localization signal (NLS). NLS does not exist in Bag-1M which is cytosolic but can be translocated to nucleus via only companion proteins and Bag-1S which always presents in cytosol. N-terminus of Bag-1L and Bag-1M consist of eight TXSEEX repeats. It is shown that these repeats have a role in DNA binding and transcription activation depending on their nuclear localizations. Having less repeat sequences, Bag-1S is the shortest isoform in the cells.

Bag-1 has many critical roles in the cell through its interactions with certain target molecules to regulate carcinogenesis, differentiation, motility, proliferation, cell survival, apoptosis and transcription. Nuclear hormone receptors, Siah, CHIP, HGF receptor, Hsp70/Hsc70 complex, Bcl-2, and C-Raf (Raf-1) are known interacting partners of Bag-1.

Cell survival and apoptosis are the two major pathways that Bag-1 is involved in. Bag-1 interactions with C-Raf or Bcl-2 are main mechanism that determine cell fate. Through these interactions Bag-1 helps to regulate the balance between cell death and cell survival. Although Bag-1 involvement in a great number of mechnanisms is known, the molecular details of these mechanisms and crosstalk between the pathways are not clear yet. The exact mechanism by which BAG1 inhibit apoptosis is unknown.In this study, we aim to understand how Bag1's expression level affect Raf1 signaling proteins and some anti-and pro-apoptotic proteins that Bag-1 found to be influencing

İNSAN MCF7 MEME KANSERİ HÜCRE HATTINDA BAG1 GEN İFADESİNDEKİ DEĞİŞİMİN ETKİLERİNİN ARAŞTIRILMASI

ÖZET

Bcl-2 asosiye athano gen (Bag) ailesi (Yunanca ölüm karşıtı athanostan) proteinleri anti-apoptotik Bcl-2 proteini ile ko-immunopresipitasyon sonucu bulunmuşlardır. Bag genleri evrimsel olarak korunmuştur ve mayalarda, bitkilerde ve hayvanlarda homologları mevcuttur. Bag ailesi üyeleri iki farklı yolla hücre homeostazını kontrol eder: (1) Hem pozitif hem negatif şekilde Hsp70/Hsc70 moleküler şaperon sisteminin çalışma döngüsünü regüle ederek hücrenin yaşamsal faaliyetlerini normal ve stres koşullarında sürdürmesine katkıda bulunur. Buna spesifik bir örnek, hücrenin normal ve stres halleri arasındaki dengesinin yönünün Bag-1'e Hsp70 ya da Raf-1'in yarışmalı bağlanması sonucu sağlanması ile verilebilir. (2) Apoptoz, tümör oluşumu, nöronal farklılaşma ve hücre döngüsü gibi birçok fizyolojik süreci kontrol eden transkripsiyon faktörleri ve reseptörler ile Hsp70/Hsc70 sisteminden bağımsız olarak kompleksler oluşturur.

Bag ailesi üyelerinin, insanlarda, kanser, AIDS ve Parkinson gibi kompleks hastalıklarda normal hücrelere kıyasla ekspresyon düzeylerinde ve hücre içi lokalizasyonlarında değişiklikler gözlenmektedir. Dolayısıyla, bu tip hastalıkları tahlil etmede Bag proteinlerinin hücre içi ekspresyonlarını ve lokalizasyonlarını incelemeye yönelik çalışmalardan yararlanılabilir. Örneğin, kanserli meme hücrelerinde yapılan çalışmalarla Bag-1 ekspresyonunun, hastalığın seyrinin izlenmesinde prognostik bir markör olarak kullanılabileceği gösterilmiştir.

Hücrelerde Bag-1 izoformlarinin ekspresyon düzeyleri farklılık göstermektedir. Bu izoformlar hücrelerde bulunma sıklıklarına göre Bag-1S, Bag-1L ve Bag-1M olarak sıralanabilir. Buna rağmen tümörlü hücrelerde ekspresyon düzeyi en yüksek olan Bag-1L izoformudur. Bunun nedeni ise Bag-1L'nin nüklear lokalizasyonuna bağlı olarak transkripsiyon faktörlerinin aktivitesini yönlendirmesi ve böylece hücre proliferasyonunu dolaylı yoldan

tetiklemesi olabilir. Bag-1 izoformlarinin hücrelerde daha önce yapılmış çalışmalar sonucu belirlenmiş etkileşim partnerleri; Bcl-2, Raf-1, Hsc70/Hsp70 sistemi,

nükleer hormon reseptörleri (NHR), ubikuitin/proteozom mekanizması ve DNA'dır.

Bu etkileşimlerle Bag-1 izoformları, hücre büyümesi için kritik sinyal iletimi, proliferasyon, transkripsiyon gibi hücresel mekanizmalardan, hücre hareketi ve apoptoza varana kadar normal, malignan ve stres altında bulunan hücreler için önemli kontrol yolaklarında görev lmaktadır.

Bag-1 izoformları, Hsp70/Hsc70 sistemini hem pozitif hem de negatif yönde kontrol ederek sırasıyla hücrelerin efektif bir şekilde protein üretebilmesi sonucu hücre

büyümesine ya da hücrelerin protein üretiminin yavaşlaması sonucu hücre ölümüne yol açabilir. Fakat bu kritik ayarlamada, henüz Bag-1'lerin Hsp70/Hsc70 sistemine tam olarak moleküler düzeyde nasıl bir kontrol mekanizması ile etkide bulunduğu net olarak bilinmemektedir. Kritik yolakların aydınlatılması yolunda literatürde yer alan örnekler aşağıda özetlenmektedir.

Bag-1'in Hsc70 ile etkileşimi sonucu Bcl-2 ile bir kompleks oluşturduğu gözlemlenmiştir. Anti-apoptotik Bcl-2 ile Bag-1'in etkileşimi, öncelikle Bag-1'in C-Raf (Raf-1) serin/treonin kinazı ile kompleks oluşturması sonucu gerçekleşir. Bunun sonucunda hücrenin yaşam ya da ölüm kaderini belirleyecek etkileşimlerin tetiklendiği ortaya çıkartılmıştır.

Bu çalışmayla amaçlanan, MCF-7 meme kanseri hücre hattında Bag-1-ilişkili sağkalım mekanizmasının moleküler detaylarının aydınlatılmasıdır. Bu amaç doğrultusunda Bag-1'in ekspresyon seviyeleri değiştirilmiş ve hücreye etkisi incelenmiştir.

1. INTRODUCTION

1.1 General information about BAG1 and its role in cell survival and apoptosis

The oncogene BCL2 is a membrane protein that blocks a step in a pathway leading to apoptosis or programmed cell death. The protein encoded by this gene binds to BCL2 and referred to as BAG1 (BCL2-associated athanogene1). It enhances the anti-apoptotic effects of BCL2 (for b lymphoma2) and represents a link between growth factor receptors and anti-apoptotic mechanisms. BAG1 isoforms have distinst subcellular localization and anti-apoptotic functions, but the exact mechanism by which BAG1 inhibits apoptosis are unknown. BAG1 as a multifunctional protein interacts with a wide range of target molecules to regulate apoptosis, proliferarion, transcription, metastasis, and motility. BAG1 protein is expressed as three major isoforms, and one minor isoform. These four protein products are generated from a single mRNA with translation initiation from four different start codons through a leaky scaning mechanism. Isoforms of BAG1 cooperate with various other proteins and also DNA to facilitate the mentioned critical cellular processes.

Cell death plays an important role in a wide variety of physiological circumstances essentially in all complex multicellular organisms. Several genes have been identified that participate as either inducers or repressors of programmed cell death. Among these, BCL2 is a blocker of cell death that was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations found in the majority of non-hodgkins Bcell lymphoma (Sharp et al. 2004). Gene transfer-mediated overexpression of the BCL2 protein has demonstrated that BCL2 inhibits apoptosis induced by a variety of stimuli such as starvation of growth factors, Fas-stimulation, and cytotoxic T-cell killing, thus suggesting that BCL2 might function in a common pathway of apoptotic signaling for protection from cell death. So this protein regulates a distal step in a final common pathway for apoptotic cell death. Elements of this pathway appear to be well conserved throughout evolution (Takayama et al. 1995). BCL2 has been shown to bind to several homolog proteins including Bax (for BCL2 associated protein), but BAG1 lacks sequence homology with BCL2 and its related proteins, indicating that it is not a member of the BCL2 protein family. BAG1 therefore may represent the prototype of a novel type of anti-cell death gene. BAG1 contains an ubiquitin-like sequence domain located at residues 37-73, which may be

important for a site for poly-ubiquitination followed by adenosine triphosphate-dependent protein degradation. In contrast to BCL2 protein, BAG1 is ubiquitously expressed in almost all cells of different lineages. BCL2 overexpression is vitally important to its ability to block cell death. If partner proteins of BCL2 such as BAG1 are present as insufficient levels or dominant inhibitors such as Bax or BCL-xs are produced at excessive levels, a mere increase in BCL2 protein levels may be inadequate to protect some types of cells from some kinds of cell death stimuli. BAG1 expression is frequently altered in a range of human cancers relative to normal cells. The expression of BAG1 has been associated with outcome in several cancer types (Cory et al. 2002). The prognostic impact of BAG1 has been investigated predominantly in breast cancer (Van Eijck et al. 2012). Breast cancer is the most common cancer that affect women. Estrogen play an important role in the development of breast cancer. Breast cell growth both normal and abnormal is stimulated by the presence of estrogen. Estrogen is mediated by estrogen receptors through estrogen response elements (ERE) and functions as a ligand dependent transcription factor. So hormonal therapy is a very effective treatment against breast cancer with hormone receptor positive patients, which blocks the ability of estrogen to turn on and stimulate the growth of breast cancer cells.

Different BAG1 isoforms have distinct functions in modulating survival in breast cancer cells (Jun Chen et al. 2009). The anti-apoptotic function of BAG1 isoforms may be mediated through decreased BCL2 protein degradation (Liu et al. 2009). BAG1 and BCL2 act as a couple in ER positive breast cancer cells and cooperate in the regulation of apoptosis. Many growth factors are known to provide not only proliferative stimulus but also anti-apoptotic stimulus simultaneously through the ligand-specific receptors. Findings that BAG1 and BCL2 expression is regulated by the same signaling pathway in cytokine stimulation imply the functional significance of the cooperative expression of these two anti-apoptotic gene products (Youle et al. 2008). BAG1 is constitutively expressed at low levels even without cytokine stimulation, whereas the level of BCL2 expression is under the detectable level (Wang et al. 1996). Overexpression of BAG1 alone could not prolong cell survival of a human T-cell line upon Fas-stimulation, but co-expression of both BAG1 and BCL2 did enhance resistance to apoptosis more than overexpression of BCL2 alone. So the synergistic effect of the BAG1 and BCL2 expression strongly suggests that the cooperative expression may be required for proceeding cell survival.

Cancers are believed to arise from a series of sequential mutations that occur as a result of genetic instability and/or environmental factors. It is known that normal stem cells have mechanisms that make them relatively resistant to chemotherapy, such as increased expression of BCL2 family proteins, increased expression of membrane transporters like breast cancer drug resistance protein, and multiple drug resistance. The expression of such proteins in tumorigenic breast cancer cells may make them inherently more resistant to current therapies. High levels of cytoplasmic BAG1 expression are detected in two thirds or more cases of breast cancer (Liu et al. 2009). BAG1 may be phosphorylated under some conditions (Cato et al. 2001), but the functional significance of this and other potential post-translational modifications such as BAG1 ubiquitylation (Sourisseau et al. 2001) are poorly understood. BAG1 overexpression in cancer may arise from hyperphosphorylation and acetylation of histones via the recruitment of 14-3-3 and histone acetyltransferases. Thus, the expression of BAG1 can be regulated by epigenetic mechanisms in nucleus. Further analysis of BAG1 promoter showed that it has a 272 bp long CpG island located between nucleotide positions 7472-7200, indicating that expression may be regulated by methylation/demethylation. Mostly, oncogenes are activated by DNA demethylation and this goes into a further assert that demethylation of BAG1 promoter could contribute to the BAG1 overexpression in cancer cells.

Despite the interesting and encouraging results obtained from studies of BAG1 in breast cancer, the ability of BAG1 overexpression to promote tumour formation in transgenic animals is yet to be tested. Such studies may produce compelling evidence that BAG1 plays a role in cancer development.

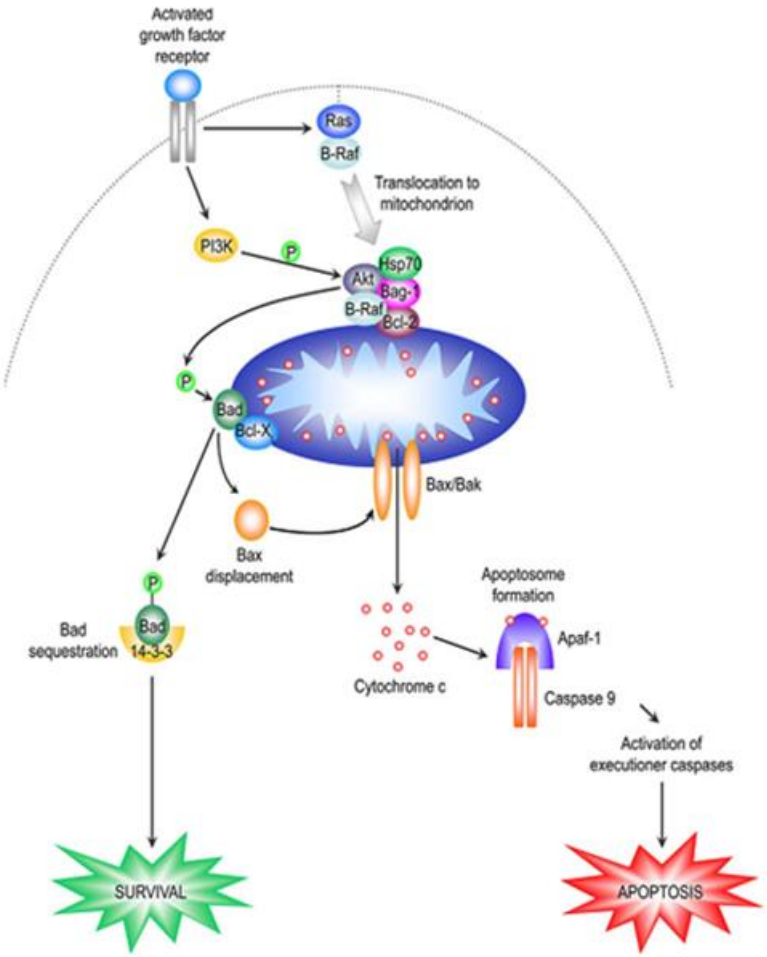


Figure 1.1: A possible model of Raf-1, BAG1 and 14-3-3 regulation in cell survival and apoptosis, BAG1 has been suggested to act as a scafold protein to promote Bad phosphorylation and Raf-1 activation in neurons (Wood et al. 2009).

1.2 BAG1 stucture in detail

The BAG1 (Bcl-2 associated athanogene gene) is located on human chromosome 9, band 13.3 a consisting of 7 exons (Takayama et al.1995). Its transcription may be initiated at one of the four promoters. The promoter has been dispersed along the positions -353 to -54 upstream of the first translational start codon and is a binding site for several transcription factors (Takayama et al. 2005). Translation at the internal AUG start site produce a major human BAG1 isoform: BAG1S, 230 aa. 36 kDa. Alternative translational initiation start sites result in both longer forms of the

protein termed BAG1L, 50 kDa and BAG1M, 46 kDa, which initiate at upstream (66th) CUG and (279th) AUG codons, respectively, and shorter form or minor isoform 26 kDa at (504th) codon (since it is very scarce in the cell and thus is not consistently detected) (Gehring et al. 2006). Even though alternative translation mechanism is not yet clear, there are hypothesis that might explain for the mechanism of translation (Townsend et al. 2005). While Bag1L and Bag1M isoforms are translated via of 5' cap structure of Bag-1 mRNA (Coldwell et al. 2001), Bag-1S isoform translation is dependent on an internal ribosome entry site (IRES). The ribosome binds to IRES element of the mRNA (Dobbyn et al. 2008).

Various domains have been identified within BAG1 proteins, termed BAG domain, ULD domain and NLS domain, which are found in closely related proteins. BAG1 protein is a homodimer but forms a heteromeric complex with HSP70/HSC70. Subcellular localization of Bag-1 isoforms is important for different biological mechanisms (Knee et al. 2001). The NLS (nuclear localisation signal) or (nuclear localisation sequence) domain of BAG1 mRNA is responsible for nuclear localization of BAG1L (Knee et al. 2001). BAG1M has both nuclear and cytosolic fractions of NLS according to the initiation site of translation of the mRNA (Brimmell et al. 1999). However, the localization of BAG1 isoforms can be regulated by cellular stress conditions which can cause relocalization of BAG1S and BAG1M to the nucleus (Townsend et al. 2002). These isoforms have different impacts on heat shock protein function, ubiqutination machinery and different transcriptional activities (Liu et al. 2009). C- terminal "BAG domain" is found in all members of BAG protein family. BAG domain was first described as a conserved region which has about 50 amino acids (Takayama et al. 1999), but recent studies with advanced techniques like X-ray crystallograpghy, and multidimensional nuclear magnetic resonance (NMR) revealed that BAG1 domain has 110-124 amino acids and it consists of three anti-parallel α-helices, each approximately 30-40 amino acids in length (Briknarova et al. 2001). These helices provide interaction sites for other proteins; the first and second helices (α-1 and α-2) interact with the serine/threonine kinase Raf-1 and the second and third helices (α-2 and α-3) are the sites of interaction with the ATPase domain of Hsc70/Hsp70 (Takayama et al. 2001) and TNF-R1 (Antoku et al. 2001). As mentioned all three isoforms of BAG1 has a ubiquitin-like domain (ULD) (Bricknarova et al. 2001). Ubiquitin is a ubiquitous 76

amino acid protein that is covalently attached to protein substratess to target proteins for degradation by proteosome, the major non-lysosomal proteolytic complex in cells (Pickard et al. 2001). BAG1 has been shown to be ubiquitylated, but BAG1 proteins have relatively long half-lifes (Luders et al. 2000). Therefore BAG1 may play a role in coordinating ubiqıitin system instead of participating in conjugation reactions (Townsend et al. 2003). Although the ULD of BAG1 is structurally related to ubiquitin, BAG1 is not covalently attached to protein substrates. The ULD is essential for some but not all biologic functions of BAG1, and does appear to be significant for binding to the proteasome (Townsend et al. 2005).

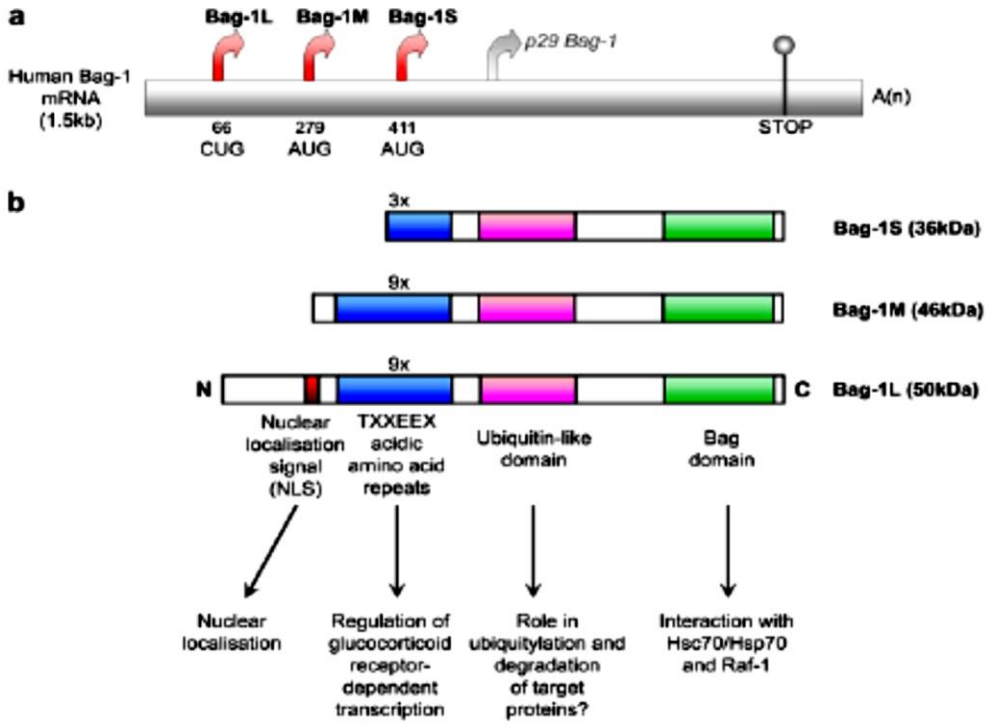


Figure1.2 : Schematic presentaiton of Bag-1 mRNA. Alternative translation sites of Bag-1 mRNA (A). Bag-1 isoforms with the binding domains (B) (Zheng et al. 2010)

1.3 BAG1 function in detail

BAG1 exhibits positive expression in many malignant tumors as an anti-apoptotic protein. BAG1 is multifunctional and the function of its individual isoforms may depend on both cell context and expression level.

All BAG1 isoforms by binding to BCL2 and heat shock protein 70s (Hsp70s) can modulate the function of these proteins in the cytosol, and also they bind to nuclear hormone receptors to inhibit hormone-induced apoptosis in the nucleus. BAG1 isoforms and their subcellular location, thus their interaction partners are tissue specific. This might explain the variable effect of BAG1 protein on the outcome in different tumours. Different BAG1 isoforms have distinct functions (Jun chen et al.2009).In general, though, overexpression of BAG1 supresses activation of caspases and apoptosis (Hill et al. 2004). BAG1 can independently associate with Raf-1 and/or BCL2 and provide at least two potential mechanisms to promote survival (Song et al. 2001). Heat shock proteins are also required for cell survival and direct activation of these proteins may also be important in the pathways that modulate survival. Supression of apoptosis might contribute to the ability of BAG1 to promote metastatic spread (Yawata et al. 1998).

BAG1 potentiatiates the ability of BCL2 to inhibit apoptosis in breast cancer cells, but the exact mechanism by which BAG1 co-expression with BCL2 is currently unknown. BAG1 may affect the function of BCL2 by modulating its expression either at the transcriptional or post–translational level. The increased expression of BCL2 in cells transfected with BAG1 suggests that BAG1 function may be required to inhibit BCL2 protein degradation (Liu et al. 2009). BAG1 may also function by directly influencing apoptosis of breast cancer cells in a BCL2-dependent manner. BCL2 proteins are thought to function, at least in part, by inserting into membranes and contributing to the formation or regulation of channels. It has been hypothesized that BAG1 may alter BCL2 function by acting on heat shock proteins which may facilitate conformational changes of BCL2 itself or proteins which might pass through the BCL2 dependent channels (Jill et al. 2012). Thus, breast cancer cells may be effectively get protected from apoptosis by a relatively low level of BCL2 in conjugation with significant BAG1 expression. In addition, BAG1 binds and modulates function of hepatocytes growth factor receptor which may also be important in breast carcinogenesis and represent another potential target of BAG1 in breast cancer.

A second key function of BAG1 in cancer is the regulation of NHRs. BAG1 potentiate activity of estrogen receptors (ER) which mediates proliferative, and survival responses to estrogen in hormone dependent breast cancers (Sharp et al. 2004). The androgen receptor (AR) is important in prostate cancer and BAG1

increases the sensitivity of AR expressing cells to androgens. BAG1 also modulates the activity of the vitamin D3 receptor (VDR) and represses the activity of retinoic acid receptor (RAR), thyroid receptor (TR) and glucocorticoid receptor (GR) (Cato et al. 2001). The effects of BAG1 on some nuclear hormone receptors requires the BAG1 carboxy terminus and it is possible that NHR interactions of BAG1 involves chaperone molecules which are known to be important for NHR function (Takayama et al. 1998). This has been demonstrated most clearly for the AR. Effects on NHR may be partly through BAG1 triggered conformational changes mediated by heat-shock proteins which may be important for altering hormone binding capacity or affinity. This is not the case for all NHRs. Biochemical and mutagenic analysis therefore suggest that there may not be a single mechanism to account for BAG1s modulation of nuclear hormone receptor activity (Cato et al. 2001). Although all BAG1 isoforms can interact with chaperone molecules and poss anti-apoptotic activity, regulation of NHR is frequently specific for the BAG1L and/or BAG1M proteins. For example, only BAG1L regulate the AR (Froesch et al. 1998) and ER (Cutress et al. 2002) whereas both BAG1L and BAG1M regulate GR. It is not clear whether the frequent specificity for the larger BAG1 isoforms stems from the nuclear localisation of these proteins or from a requirement for additional Hsc/Hsp70 independent functions encoded by the amino-termini of these isoforms. For instance, alternatively, non-specific DNA binding mediated by basic amino-acid residues of the N-terminal NLS and/or transactivating activity of the larger BAG1 proteins might play a role in the stabilization of the receptor, DNA complexes and/or recruitement of transcription factors (Zeiner et al. 1999).

Although Bag-1 predominantly functions to suppress apoptosis, most likely acting between the growth/survival receptors and the mitochondria, it also involves other regulatory points in the apoptotic cascade (Takayama et al. 2001). Independent of NHRs, BAG1 isoforms can directly interact with Hsc/Hsp70 chaperones to function as their nucleotide exchange factor. By the synergistic action of these proteins, efficient folding can be achieved in the cell. Simple regulation of the refolding action of Hsp70s by BAG1 is not sufficient for all the observed actions of BAG1, since other BAG1 protein regions are required for its biological functions. BAG1 may act as a scaffold molecule, to functionally link chaperone function with specific target molecules (Hohfeld et al. 2001). One of the interactions of BAG1 that may not occur through direct HSC70/HSP70 binding is with Raf-1. It is found in some of the

reports that Raf-1 and Hsp70 competitively bind to a similar site on BAG1 (Sondermann et al. 2001). Since, though, some studies proved the otherwise, this contreversy needs to be clarified. It could be possible that Hsp70 levels would be important to determine the interaction profile of BAG1. High levels of HSP70 that accumulate in stressed cells may displace Raf-1 and this shuts down the important signaling path that promote cell survival and proliferation. Therefore, BAG1 isoforms may act as a molecular switch in signaling pathways that direct cells toward different states depending on whether enviromental conditions are hospitable or stressful (Van Eijck et al. 2012).

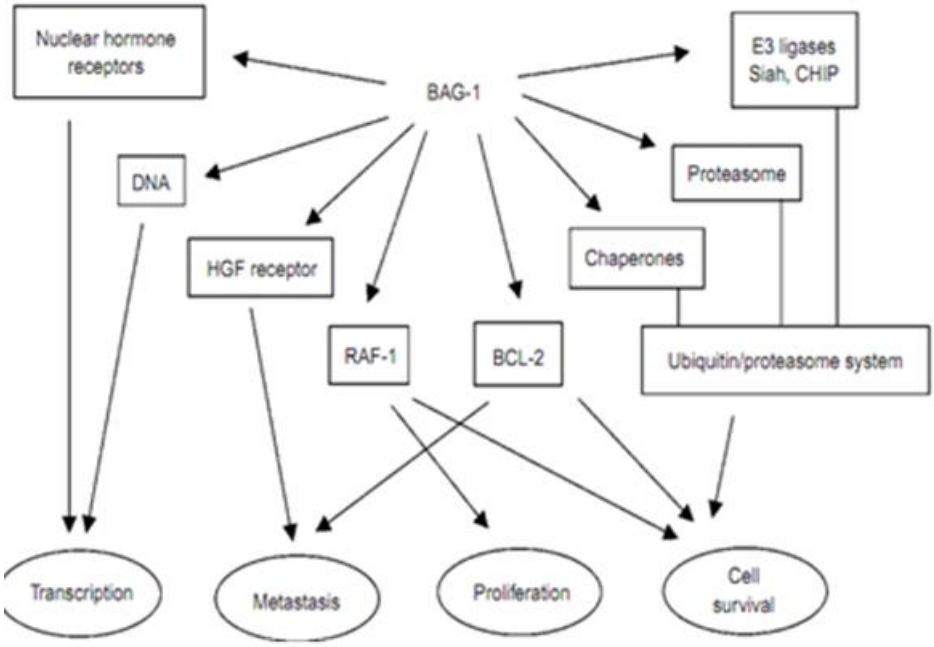


Figure 1.3: BAG1 interaction partners and their molecular mechanisms

1.4 RT-PCR

1.4.1 An introduction to real-time PCR

There are several ways to question a cell for changes induced by artificial or natural agents during a biological process. One way is to look for changes in cellular transcript levels that may indicate changes in the corresponding proteins, or looking for an increase in the level of expression from a transgene or the inhibition of expression of an endogenous gene by a siRNA is the question of interest.

Quantitative real-time PCR technology can be utilized to provide the required information.

1.4.2 A brief history of nucleic acid detection and quantification

Initially, nucleic acid quantification meant the addition of radiolabeled UTP or deoxythymidine triphosphate (dTTP) to cell cultures or one of many possible in vitro experimental preparations and measuring their incorporation into nucleic acids by TCA (trichoroacetic acid) precipitation. It was now possible to specifically identify a particular gene or transcript by hybridization of a radioactively labeled probe to a membrane bearing DNA restriction fragments or RNA.

In combining the reverse transcriptase (RT) reaction with the PCR, identification of a specific RNA transcript was now possible from very low copy numbers of starting material. Quantification of transcripts from sample unknowns became possible with the advent of competitive RT-PCR (Vu et al. 2000). Known amounts of the RNA product were spiked into the RT reaction and converted into cDNA along with the target sequence within the unknown sample. Subsequently, both the truncated standard and unknown target sequences were amplified using the PCR. The products on the gel were imaged following staining with ethidium bromide or SYBR Green I. Real-time quantitative PCR has become the most accurate and sensitive method for the detection and quantification of nucleic acids yet devised. This method has overcome most of the major shortcomings of the preceding ones. Using the specificity and sensitivity of PCR combined with direct detection of the target of choice utilizing fluorescently labeled primers, probes or dyes, the inherent problems of gels, transfer to a membrane, radioactive probe hybridization and the limitations of film as a detector have been eliminated. There are two problems for real-time PCR that still linger. They are 1) methods of quantification, *i.e.* kind of standard, assay quality and calculation methodology used and 2) how to properly normalize different samples to correct for differences in nucleic acid input from sample to sample.

1.4.3 Real-time quantitative PCR

Real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles. Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample.

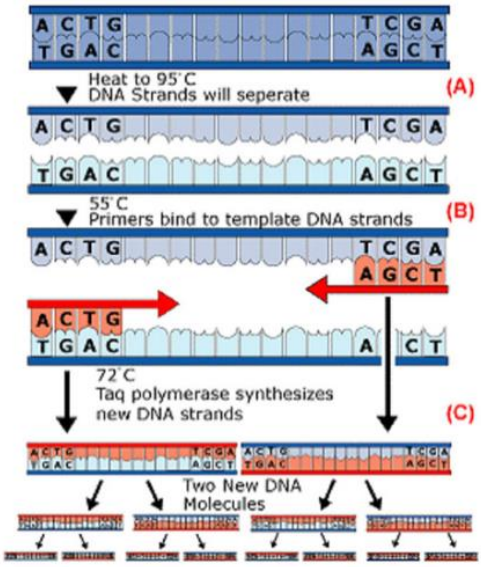


Figure 1.4: PCR starts with the denaturation of double strand DNA. After denaturation, primers anneal to their complementary sequences on single strands. Synthesis of the new strands is catalyzed by special polymerases. In every reaction turn, two daughter double-stranded DNA is produced from the parent strand.

1.5 Aim of the study

In this study, we aimed to elucidate the molecular mechanism of Bag-1-associated cell survival in MCF-7 breast cancer cells.We comprehensively investigate BAG1 mediated changes in gene expression level by comparing the gene expression profile after BAG1 siRNA silencing in MCF7 human breast cancer cell line. To understand Bag-1 and its isoforms involvement in anti-apoptotic processes in MCF-7 breast cancer cells, we silenced Bag-1 gene using Bag-1 siRNA. We first analyzed Bag-1 silencing effect on different apoptotic genes by qRT-PCR. Bag-1 silencing caused a decrease in Akt gene expression which is as a key gene for survival and also decreased Bcl-2, C-Raf, B-RAF and 14-3-3theta mRNA expression levels. Earlier immunoblotting results showed that Akt activation, which is a marker for cell survival, was lost by Bag-1 siRNA transfection, revealing complementarity between mRNA and protein levels. Studies on protein level analysis for C-Raf and Braf showed a significant downregulation of these proteins after Bag-1 silencing. We also studied the alterations on other pro-and anti-apoptotic proteins expression level with Bag-1 silencing to further understand the molecular details of BAG-1 involved apoptotic mechanism. We believe that once the molecules in the BAG-1 related apoptotic pathway is delineated, Bag-1 might be used as a marker for cancer studies

both for treatment and prognosis. our results seggest that BAG1 could be a target for cancer treatment.

2. MATERIALS and METHODS

2.1 Materials and equipment

2.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A.

2.1.2 Compounds, kits and buffers

The compounds and commercial kits used during the study are listed in Appendix B; the recipes of buffers and media used in the experiments are given in Appendix C.

2.2 Methods

2.2.1 Preparation of cell culture

MCF-7 is a human breast cancer cell line that was first isolated in 1970 from the malignant adenocarcinoma breast tissue of a 69-year old woman. MCF-7 is the acronym of Michigan Cancer Foundation, referring to the institute in Detroit where the cell line was established. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen via estrogen receptors. MCF-7 cells are also sensitive to cytokeratin. When grown in vitro, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can also be inhibited using tumor necrosis factor alpha (TNF alpha).

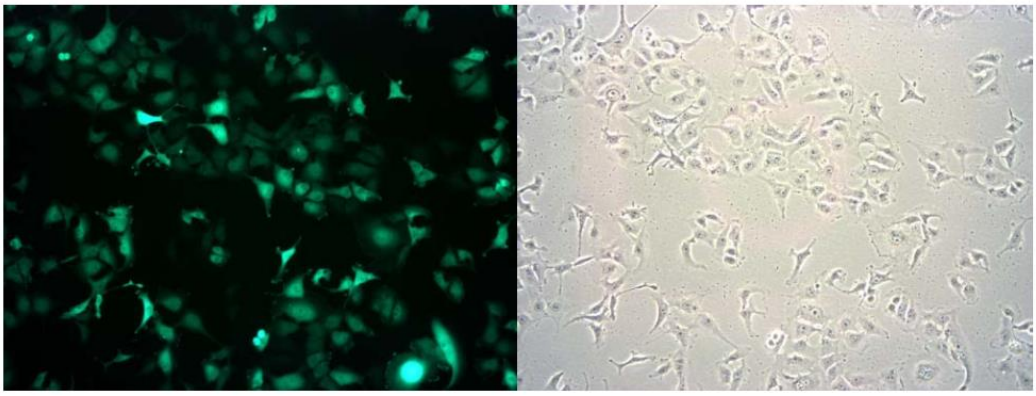


Figure 2.2.1 MCF-7/GFP Cell Line. Left: GFP fluorescence; Right: Phase contrast (Biolab research 2012)

Quality Control

This cryovial contains at least 1.0×10^6 MCF-7/GFP cells as determined by morphology, trypan-blue dye exclusion and viable cell count. The MCF-7/GFP cells are tested free of microbial contamination.

2.2.1.1 Establishing MCF-7 Cultures from Frozen Cells

Place 10 mL of complete DMEM growth medium in a 50 mL conical tube. Thaw the frozen cryovial of cells within 1–2 minutes by gentle agitation in a 37°C water bath. Decontaminate the cryovial by wiping the surface of the vial with 70% (v/v) ethanol. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium. Collect the cells by centrifugation at 1400 rpm for 3 minutes at

room temperature. Remove the growth medium by aspiration. Resuspend the cells in the conical tube in 15 mL of fresh growth medium by gently pipetting up and down. Transfer the 15 mL of cell suspension to a T-75 tissue culture flask. Place the cells in a 37°C incubator at 5% CO2. Monitor cell density daily. Cells should be passaged when the culture reaches 95% confluence. Cultures need to be initiated with the same number of cells, therefore population density will effect the behavior of the cells in the same manner. According to this, the cells were seeded after counting. The useful numbers for cell culture shown in Figure: 2.2.2. There are various sizes and dishes and flasks used for cell culture. Some useful number such as surface area and volumes of dissociation solutions are given here.

	Surface Area (mm^2)	Seeding Density	Cells at Confluency[1]	Versene (ml of 0.53 mM EDTA)	Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA)	Growth Medium (ml)
Dishes						
35 mm	962	0.3×10^6	1.2×10^6	1	1	2
60 mm	2,827	0.8×10^6	3.2×10^6	3	2	3
100 mm	7,854	2.2×10^6	8.8×10^6	5	3	10
150 mm	17,671	5.0×10^6	20.0×10^6	10	8	20
Cluster Plates						
6-well	962	0.3×10^6	1.2×10^6	2	2	3–5
12-well	401	0.1×10^6	0.4×10^6	1	1	1–2
24-well	200	0.05×10^6	0.2×10^6	0.5	0.5	0.5–1.0
Flasks						
T-25	2,500	0.7×10^6	2.8×10^6	3	3	3–5
T-75	7,500	2.1×10^6	8.4×10^6	5	5	8–15
T-160	16,000	4.6×10^6	18.4×10^6	10	10	15–30

[1] The number of cells on a confluent plate, dish, or flask will vary with cell type. For this table, HeLa cells were used.

Figure 2.2.2. Useful number of the cells on a confluent flask.

2.2.1.2 Cell counting

Growing medium was removed and cells were detached by 1 mL Trypsin-EDTA treatment for 2 min at 37°C. Then trypsin-EDTA solution was inhibited by 10 mL medium and cells were suspended Then 10 µL suspension was taken and put onto the hemocytometer or bright line counting chamber, cells were counted as the number of cells per 25 square (1 mm^2).

2.2.1.3 Hemocytometer method of cell counting

The hemocytometer cell counting method uses a specially crafted gridded-glass slide consisting of two chambers, each divided into nine 1 x 1 mm squares. A glass cover is supported by the grid, and the cell suspension is loaded via capillary action into the chamber. The cells lie over the grid occupying a volume of 0.1 µL for each 1 x 1 mm square, and are counted manually. The number of cells counted in a specific number

of squares and the dilution factor are used to calculate the original concentration in cells/mL. Proper sample preparation technique is critical when working with the traditional hemocytometer. The Glass hemocytometer, with its free cover slip, is subject to over-filling, resulting in cell numbers that are often higher than those produced by disposable, fixed cover slip hemocytometers. Careful, consistent sample loading must be performed to avoid over-filling. The cell suspension must also be properly diluted so that a reasonable number of cells (25 to 250) are placed in each square. The subjective nature of human manual counting can lead to wide variation in results. In addition, the procedure is tedious and requires careful cleaning and handling of the hemocytometer parts.

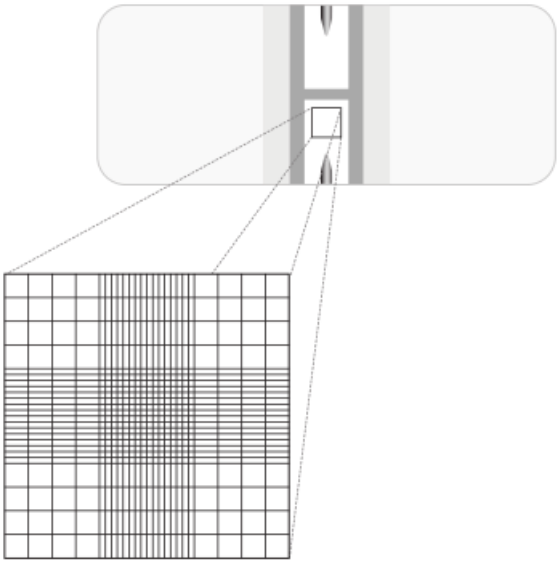


Figure 2.2.3: chamber structure and cell counting process.

The cell number calculation is given below:

The number of cells per cubic millimeter:

Number of cells counted per square millimeter x dilution if used x 10

The number of cells per millimeter:

Number of cells counted per square millimeter x dilution if used x 10,000

2.2.1.3 Cell passage

After cells were lifted and cell density was determined (%80 and above), cells were put into the centrifuge tubes and centrifuged 1400 rpm for 3 min. The supernatant

was discarded and pellet was resuspended with culture medium and $1x10^6$ cells were transferred to clean culture flask T75.

2.2.1.4 Cell freezing

After cell passage, cells resuspended in medium were transferred to freezing tube with freezing medium for future experiments. Estimated cell number for per freezing tube was $1x10^6$ cell/mL.

2.2.2 siRNA transfection

Lipofectamine™2000 has been used successfully to transfect short interfering RNAs (siRNA) into mammalian cells for RNA interference (RNAi) studies (Gitlin *et al.*, 2002; Yu *et al.*, 2002).

Transfection conditions are provided for a MCF7 cell lines as a starting point, then we optimizing transfection conditions to obtain the best results for your target gene and siRNA. Santa Cruz human BAG1 siRNA was used for the silencing of Bag-1 gene. Lyophilized siRNA stock was 330 µL RNAse-free water and diluted as a working solution for transfection. 40 pmol dilution of siRNA was optimum for MCF-7 cells to silencing.

Opti-MEM® Reduced Serum Medium was used for dilution of siRNA to form transfection comlex. Since Opti-MEM® Reduced Serum Medium is an improved Minimal Essential Medium (MEM) that allows for a reduction of Fetal Bovine Serum supplementation by at least 50% with no change to growth rate or morphology and it is also recommended for use with cationic lipid transfection reagents, such as Lipofectamine reagent, it was chosen as transfection medium. Lipofectamine 2000 of Invitrogen was used as lipid agent to increase permeability of cell membrane, and instructions of the manufacturer were followed.

2.2.3 Survival Assay (Trypan Blue Dye Exclusion Assay)

Trypan Blue is a vital dye of which reactivity is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. According to the time points, cells were counted each day for 4 days using dye exclusion assay. $0,5x10^5$ cells were seeded each well of the 6-well plate one day before transfection. 24, 48, 72 and 96 h after transfection, growth medium was removed and cells were washed with PBS, twice. Cells were trypsinized

and gathered by centrifugation. Then, cells were exposed to 0.4% (w/v) Trypan blue dye (50 µL) and DMEM (50 µL) at 1:1 ratio. Dual-chamber FisherLab hemacytometer was filled with 10 µL of cells from trypan blue-DMEM mixture, and under a light microscope, non-viable cells were observed as stained while viable cells excluded the stain. Stained cells were counted and plotted.

2.2.4 Primer design

Optimal design of the PCR primers is essential for accurate and specific quantification using real-time PCR. Primer detection is based on the binding of the SYBR GreenI dye into double-stranded PCR products, which is a sequence-independent process. Primer design is very restrictive: the annealing temperature is limited to between 55 and 60°C, which correspond to the optimal working conditions for the Taq DNA polymerase enzyme, and the length of the PCR product has to be set between 80 and 150 bp. In the first step primer designed to determine the sequence that needs to be targeted by the PCR. And in The second step related genes identify and eliminate regions of high homology, which may further restrict the length of the sequence available. A third step in primer design for cDNA quantification requires the design to overlap exon–exon boundaries.

2.2.4.1 Step by step primer design: ß-actin for a cDNA quantification assay

Identify the target sequence Browse the human genome project database with ß-actin as the keyword to identify the sequence, chromosome location (7p22.1), mRNA accession number (X00351), mRNA length (1761 bp) and gene structure (5 exons). To choose a suitable accession number to base primer design on, use the graphic representation to select an mRNA that includes all the exons and has 5’ and 3’ sequence. restrict this sequence with regards to regions of homology with related genes (a-actin, γ1 and γ2-actin) Align these sequences to determine domain of homology. Use the alignment tools on the NCBI website (BLAST 2 sequences) to find homologies.

2.2.4.2 Determine exon-exon boundaries

Use the human genome browser, click on the accession number for the human mRNA corresponding to the full-length transcript (e.g. X00351). Click on the accession number at the top to display the sequence database information. Click on the accession number under ‘query’ in the mRNA/Genomic alignment section to

obtain 5’ and 3’ sequences, coding region, ATG and STOP codons, exon–exon boundaries. Avoid inconsistent splicing sites and polyadenylation signals.

2.2.4.3 primers Design

As a result the sequence available for designing primer to quantify the expression of ß-actin is restricted to two regions, between nucleotides 1 and 90 or between nucleotides 1208 and 1763. There is no exon boundary in the second region. The design should, therefore, be limited to the region between the first and second exons of the gene. Transfer this sequence to the primer design software (e.g. Primer Express from ABI) and proceed to design.

Copy the mRNA sequence into a simple PCR document. Restrict the target sequence accordingly. Select the option for primer Tm (59°C) and amplicon length (80 to 120). Keep the CG content at default settings and primer length below 24 bp. See if the software comes up with a possible primer pair. If not, use the primer test document and try to select sections of sequence manually. This sequence is very CG rich, therefore, select a region with A or T as 3’ for each primer. Elongate the primer in 5’ until reaching a Tm between 58° and 60°C. Lastly, BLAST the primers (limit to the human species and mRNA bank). If the restriction in the target position has been done thoroughly, the BLAST search should give no matches. Quite a lot of hits are nevertheless reported for these primers. Most of them are on anti-sense strands of cDNAs. If none of them comes in both the forward and the reverse primer BLAST searches, no PCR product can be generated from these hits. These primers are located in the 5’ of the mRNA.

Table 2.1: Primer design based on sequence alignment.

PRIMER SETS:
1.Name of the oligonucleotide: **BCL2 variant alphamRNA-F**
Sequence (5' to 3'):
TCCCTCGCTGCACAAATACTC
2.Name of the oligonucleotide: **BCL2 variant alphamRNA-R**
Sequence (5' to 3'):
ACGACCCGATGGCCATAGA
3.Name of the oligonucleotide: **BAX variant alphamRNA-F**
Sequence (5' to 3'):
CCAAGGTGCCGGAACTGA
4.Name of the oligonucleotide: **BAX variant alphamRNA-R**

Sequence (5' to 3'):
TCCCGGAGGAAGTCCAATG
5.Name of the oligonucleotide: **BAD variant 1mRNA-F**
Sequence (5' to 3'):
GAGTGACGAGTTTGTGGACTCCTT
6.Name of the oligonucleotide: **BAD variant 1mRNA-R**
Sequence (5' to 3'):
TGTGCCCGCGCTCTTC
7.Name of the oligonucleotide: **GAPDH variant 1 Mrna-F**
Sequence (5' to 3'):
ATGGAAATCCCATCACCATCTT
8.Name of the oligonucleotide: **GAPDH variant 1 Mrna-R**
Sequence (5' to 3'):
CGCCCCACTTGATTTTGG
9.Name of the oligonucleotide: **BAG1s.Mrna-F**
Sequence (5' to 3'):
CCCCTCATTTGTTGGCTGTCT
10.Name of the oligonucleotide: **BAG1s.Mrna-R**
Sequence (5' to 3'):
CAGTGCATCTGAGGACCTGAAA
11.Name of the oligonucleotide: **14.3.3.Mrna-F**
Sequence (5' to 3'):
GCGTGTGGTGATGATCGAAA
12.Name of the oligonucleotide: **14.3.3.Mrna-R**
Sequence (5' to 3'):
AAATGCCTCTTGGTAAGCTCCTT
13.Name of the oligonucleotide: **BRAF-F**
Sequence (5' to 3'):
CTGGCCCGCTCATTGC
14.Name of the oligonucleotide: **BRAF-R**
Sequence (5' to 3'):
CCGATTCAAGGAGGGTTCTG
15.Name of the oligonucleotide: **CRAF-F**
Sequence (5' to 3'):
TTCAGTTTCCAGTCGGATGTCTAC
16.Name of the oligonucleotide: **CRAF-R**
Sequence (5' to 3'):
CTCCCCCGTCATCAGTTCA
17.Name of the oligonucleotide: **AKT-F**
Sequence (5' to 3'):
CTGAGACGCCCGGTACATG

18.Name of the oligonucleotide: **AKT-R**
Sequence (5' to 3'):
CCCCTGCCGGTCTGAATC

2.2.5 Primer optimization

The optimization of the primer concentration is essential. Each set of primers works best at a different concentration. Primer concentration is usually determined to be optimal when the specific amplification relative to primer-dimers is maximal, in a positive versus negative control experiment. Primer optimization differs from one type of assay to another.

Table 2.2 Standard protocol for optimizing primer concentration.

	Forward primer (nM)				
Reverse primer (nM)	50	100	300	500	1000
50	50/50	50/100	50/300	50/500	50/1000
100	100/50	100/100	100/300	100/500	100/1000
300	300/50	300/100	300/300	300/500	300/1000
500	500/50	500/100	500/300	500/500	500/1000
1000	1000/50	1000/100	1000/300	1000/500	1000/1000

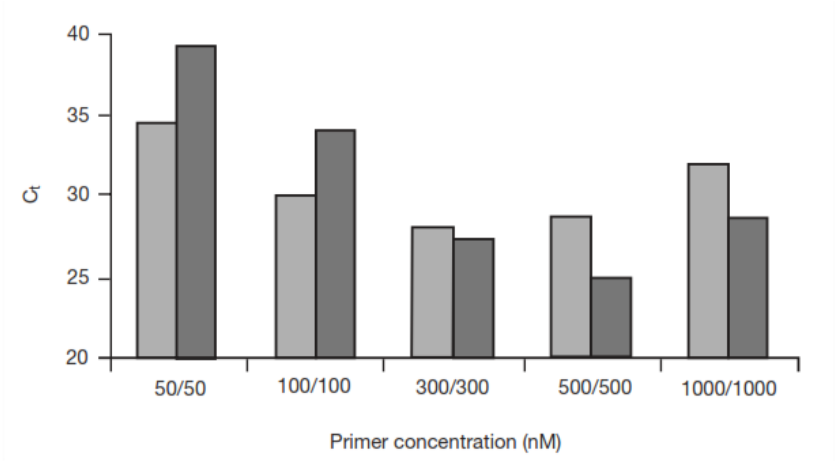


Figure 2.2.6: Two sets of primers were designed to amplify 2 gene primers at concentration 300/300 or 200/200 work better

2.2.6. Relative quantification of gene expression

This is also an easy optimization procedure, but one is looking for the best possible conditions of amplification (lowest Ct) with a similar PCR efficiency for both the target and the endogenous control(s) as reference (usually several housekeeping

genes). The procedure consists of finding the most efficient primer concentration for both genes, followed by a comparison of efficiency between target and reference. The same optimization matrix can be set up to find appropriate primer concentration conditions (both for the target and the reference genes). Then, a serial dilution of the input cDNA(1/5, 1/50, 1/500…) is used to compare the efficiency of both PCR reactions in parallel. Ct values will be different between the target and the reference but one is looking for parallelism between the two standard curves. PCR efficiencies can be compared by relating the slope of the linear curve of C values plotted against the log-dilution. A perfect reaction has a slope of 3.3, which corresponds to an exact two-fold amplification at every cycle of the PCR between dilutions of a factor 10.

2.2.7 RNA quantification, isolation and reverse transcription reaction

2.2.7.1 RNA extraction

Commercially available kits perform well enough, have the advantage of not requiring phenol extraction (with the exception of Trizol, Invitrogen Life Technologies) but are costly, we used High pure RNA isolation roche Kit. An important point is to verify the absence of genomic DNA contamination. A DNase treatment step may be included to follow RNA extraction as gDNA contamination may interfere with the assay.

2.2.7.2 Quantification of the isolated RNAs

The quantity and quality of the RNA isolation is very important for downstream steps. RNA is a very variable and unstable molecule and it is very open to the negative effects of RNase enzymes which occur almost everywhere. Therefore, it is very easy to lose RNAs during the process. Thus, we need to be sure about the healthy existence of RNAs in our tubes. On the other hand, for a standart reverse transcription reaction, same amount of sample RNAs need to be used. Therefore, a healthy measurement of these RNAs is a vital process for the fate of the study.

According to these concerns, sterile working issues had been followed especially by using RNase disinfectant solution (RNase-ExitusPlus, Applichem, Denmark) on the working areas. RNAs are measured with Nanodrop ND 1000 V 3.3 (Thermo Scientific, Wilmington, USA) spectrophotometer.

2.2.7.3 First strand cDNA synthesis

Synthesis of cDNA from mRNA is the first step of RT-PCR gene expression quantification. The quality of the cDNA is directly related to the quality of the RNA template. If possible, determine the RNA concentration of samples before using them in an RT reaction. using initiation with either oligo-dT Once prepared, first-strand cDNA should not be thawed and refrozen more than once. During PCR set-up, it has to be kept on ice and used immediately. Once the cDNA has been synthesized, it remains to be verified that no DNA contaminates the samples. As the assay to determine mRNA quantity is based on PCR, the assay to detect genomic DNA contamination also needs to be performed using PCR (an agarose gel would not be sensitive enough).

A genomic DNA target may be used as control for the size of a potential genomic DNA contamination band. The primers ATG GCAAAT TCC ATG GCA and TGA TGA TCT TGA GGC TGT TGT C for the *GAPDH* gene generate an amplicon of 285 bp from cDNA and an amplicon of 453 bp from genomic DNA.

Thaw protoscript M-MuLV Tag RT-PCR Kit components and place on ice.the 10x buffer warmed briefly at 42c and votexed to dissolve any precipitate.its important to set up a –RT control reaction,to insure there is no DNA contamination. Make the RNA/PRIMER/Dntp mix by combining the following components in a sterile Rnase free tube. Heat for 5 min at 70c .spin briefly and promptly chill on ice. Add the following components to the 16 microlitre RNA/PRIMER/dNTP solution and mix well by pipetting up and down.10x RT buffer, murine Rnase inhibitor,M-MuLV Reverse Transcriptase Nuclease-free H2o. Incubate the 20microlitre cDNA synthesis reaction at 42c for one hour. Inactive the enzyme at 80 c for 5 min. Bring the reaction volume to 50 microlitre with water. CDNA products stored at -20c.

2.2.8 Reference gene validation

A number of reference (housekeeping) genes have been described in the literature and are used at different frequencies. Suzuki and colleagues reviewed 452 papers published in 1999, in six major journals, which used real-time PCR for mRNA quantification (Suzuki et al. 2000) and found that the most frequently used reference gene was *GAPDH* (33%), followed by b-actin (32%) and 18S rRNA (14%). The importance of assay- and tissue-specific reference gene validation has also been illustrated by others (Huggett et al. 2005).The choice of a suitable reference gene is

dependent on the individual model and requires investigation of the appropriate literature.

2.2.9 Preparation of the PCR MIX

Lightcycler 480SYBR Green I MASTER KIT is ideally suited for hot start PCR applications. This kit allows very sensitive detection and quantification of defined DNA sequences.

To prepare one 20 microliter standard reaction: First we need to thaw one vial of lightcycler 480SYBR Green I MASTER and water (pcr-grade) and prepare 10x concentrated solution of the PCR primers. Later in a 1.5 ml reaction tube, one has to prepare the PCR mix by pipetting up and down (vortexing should be excluded). To start the reaction on the multiwall plate, 15 microlitres of the PCR mix should be added to each well with 5 microlitre of the DNA template. After sealing the multiwell plate with the lightcycler sealing foil, program can be run.

2.2.10 qRT-PCR conditions

qRT-PCR is performed with LightCycler 480 Real-Time PCR instrument (Roche Diagnostics, Mannheim, Germany). Reaction conditions are listed here.

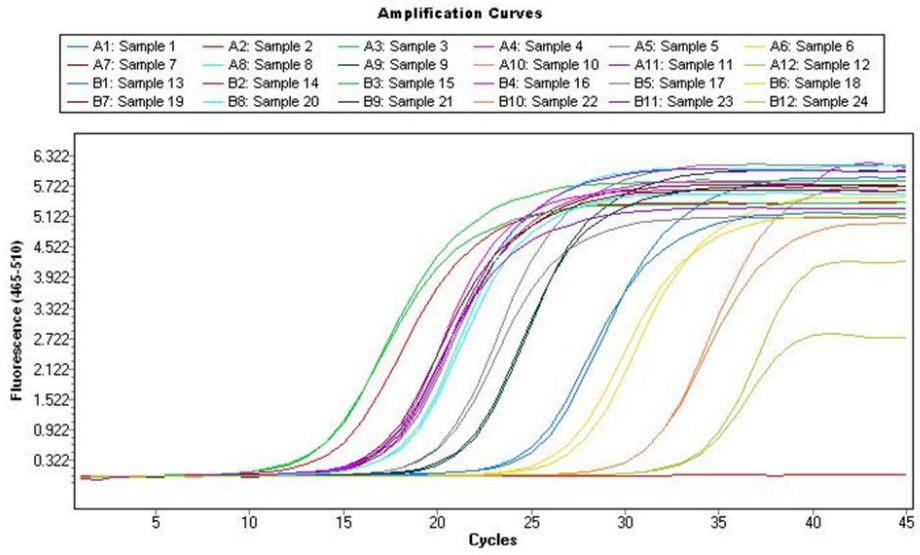
Table 2.3: Conditions of qRT-PCR.

	[^{0}C]	Time	Ramp rate [^{0}C/s]	Cycles
Denaturation	95	05 min	4,4	1
Amplification	95	10 sec	4,4	45
	55	18 sec	2,2	
	72	05 sec	4,4	
Cooling	40	10 sec	1,5	1

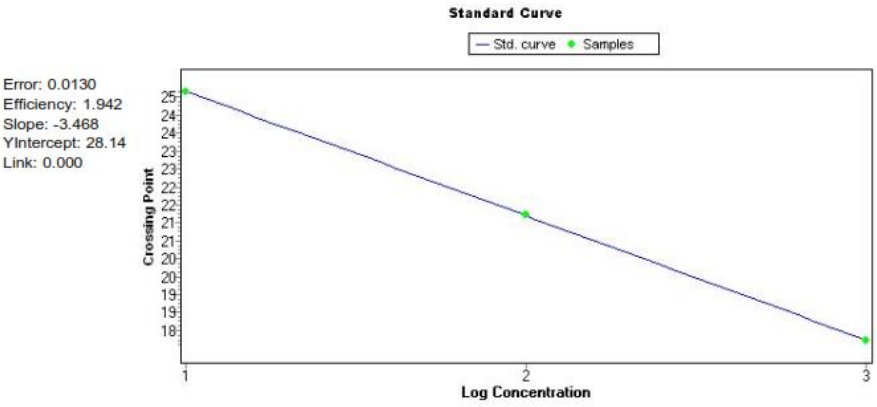
3. RESULTS and DISCUSSION

3.1 Effect of BAG1 siRNA treatment on BAG1 gene expression in MCF7 cell line

BAG1 mRNA levels were extrapolated from the standard curve generated by the amplification of serially diluted DNA standards of known concentrations of BAG1 amplicon. The measured BAG1 mRNA levels were corrected for errors arising from differences in sample preparation and reverse transcription efficiencies using the expression levels of GAPDH housekeeping reference gene as an internal standard. Representative qRT-PCR results (cDNAs used in this reaction were obtained by total RNA isolated from wild-type MCF-7 cells) are shown to demonstrate that our primer sets for both GAPDH and BAG-1 are functional. We obtained reasonable Cp values that correspond well with the dilution ratios.



A)



B)

Figure3.1: A) qRT-PCR results obtained with GAPDH primers of total RNA isolated from wild-type MCF-7 cells (red curve, Cp showing a dilution of

cDNA in 1:1 (v:v) ratio; green Cp showing a dilution of cDNA in 1:10 (v:v); purple Cp showing a dilution of cDNA in 1:100 (v:v); yellow negative control) B) standard curve (slope:-3.468, efficiency: 1.942, y:28.14)

A)

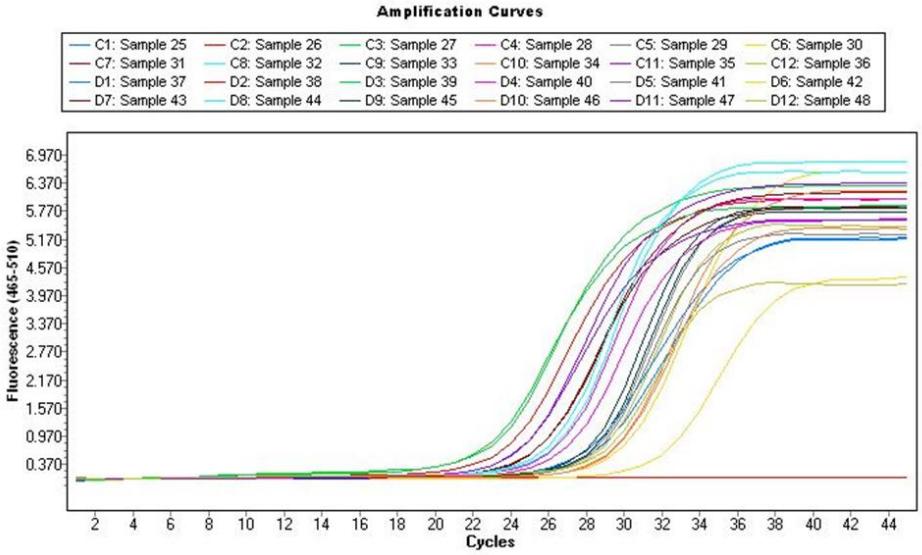


Figure 3.2 : A) qRT-PCR results obtained with BAG1 primers of total RNA isolated from wild-type MCF-7 cells (red curve, Cp showing a dilution of cDNA in 1:1 (v:v) ratio; green Cp showing a dilution of cDNA in 1:10 (v:v); purple Cp showing a dilution of cDNA in 1:100 (v:v); yellow negative control) B) standard curve (slope:-2,710, efficiency: 2,339, y:30.66)

3.3 Time-dependent BAG-1 siRNA optimization

BAG1 mRNA expression was screened in MCF7 cell lines. Aberrant or high BAG1 protein expression in MCF7 breast cancer cell line suggests it might play a role as an anti-apoptotic protein in this cell line. To address this issue, BAG1 was silenced in MCF7 cells. BAG1 siRNA was effective when administered between 24 h and 48 h (Figure 3.3).

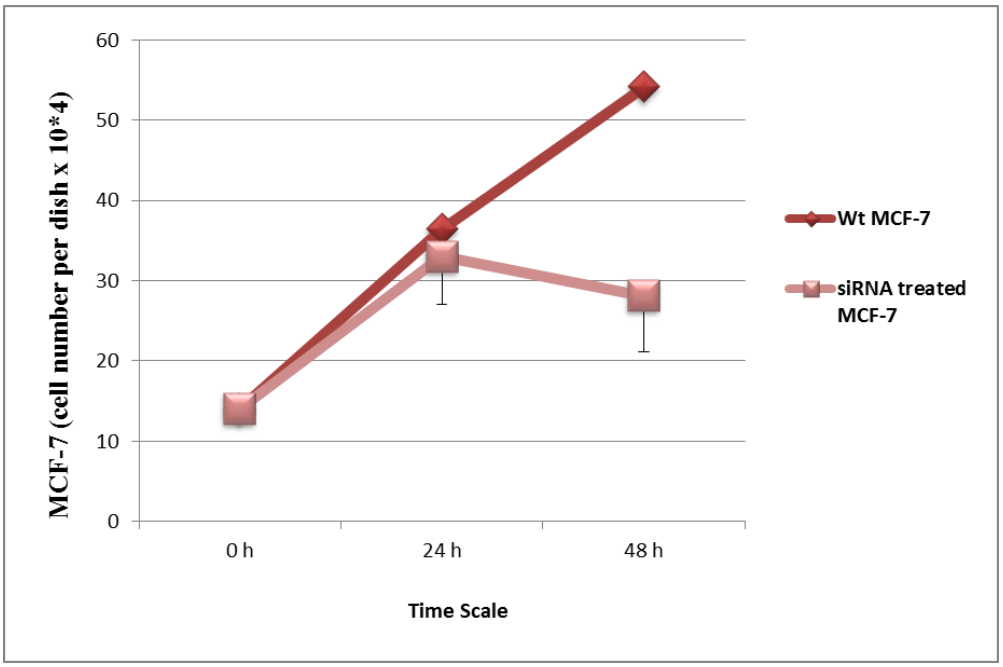


Figure 3.3 : MCF-7 cell viability gets altered due to the changes of Bag-1 expression level. Cell survival (trypan blue dye exclusion) assay revealed that viable cell numbers decreased by BAG1 silencing

3.3 BAG1 silencing affected other genes expression level in MCF7 cell line

Monitoring how other gene levels were influenced by BAG1 silencing, we found a notable decrease in BCL2, B-Raf, C-Raf, AKT, and 14-3-3 expression when treated with siRNA targeting BAG1 after 48 h. On the other hand, we observed an increase in BAD and BAX gene expression.

3.3.1 Effect of BAG1 siRNA treatment on B-Raf gene expression in MCF7 cell line

B-Raf gene (proto-oncogene B-Raf) is a serine/threonine-protein kinase. B-Raf protein is involved in cell signaling to direct cells to growth. This protein was shown to be mutated in human cancers (Wang et al. 1996**)**. Earlier studies of ours and others revealed that BAG-1 overexpression leads to the activation of Raf/MEK/ERK signaling pathway (Datta et al. 1996). In this pathway, it is observed that BAG1 forms a complex with B-Raf, C-Raf, Hsp70, BCL2 to activate the downstream players of Raf/MEK/ERK signaling pathway. So it is very interesting to study the expression profile of the proteins that BAG1 forms a complex. When B-Raf mRNA

levels were investigated after BAG1 silencing, we observed a significant decrease (mean: 0.3576, std: 0.02277, *p<0.05) (Figure 3.5). The Raf serine/threonine kinases are effectors of Ras that function as MAP3Ks in the ERK phosphorylation cascade. Mammals express three Raf proteins: Raf-1 (C-Raf); A-Raf; and B-Raf. Analysis targeting B-Raf protein levels after 24 and 48 h BAG-1 siRNA treatment also showed a similar profile; having a significant decrease in B-Raf levels (Demir et al., unpublished results). Therefore a decrease in the transcript levels correlate well with the earlier observation done on the protein levels of B-Raf.

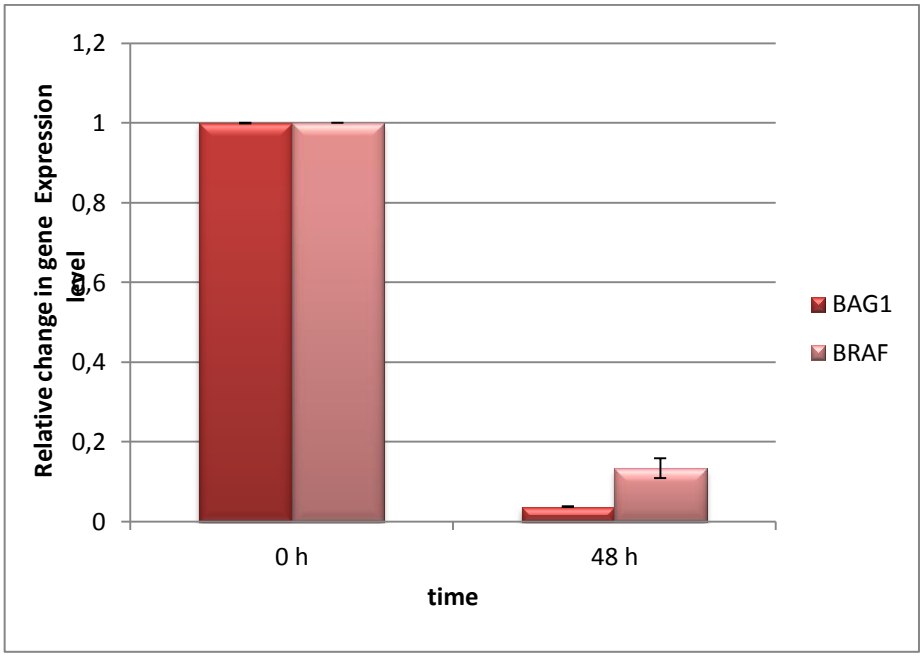


Figure 3.5: qPCR analysis of B-Raf gene after BAG-1 siRNA transfection of MCF-7 cells (mean: 20.96, std: 0.03535, *p < 0.05, 0.025)

3.3.2 Effect of BAG1 siRNA treatment on C-Raf gene expression in MCF7 cells

C-Raf (Raf-1) is a member of the Raf kinase family of serine/threonine-specific protein kinases, from the TKL (Tyrosine-kinase-like) group of kinases, and is part of the ERK1/2 pathway as a MAP kinase kinase kinase (MAP3K) that functions downstream of the Ras subfamily of membrane associated GTPases. Since BAG1 has known to interact with C-Raf under anti-apoptotic conditions, we wanted to investigate how BAG1 gene silencing affects C-Raf gene expression. We observed a significant decrease in the C-Raf transcript levels (mean: 0.06737, std: 0.05517, *p

<0.05) after Bag-1 silencing treatment was applied for 48 h (Figure 3.6). When protein levels of this protein were studied under BAG-1 silenced MCF-7 cells, we observed a dramatic decrease as well (Demir et al., unpublished results).

Our results demonstrated that the decrease in expression levels of BAG1 gene in MCF7 cancer cell line through siRNA interference process causes a decrease in C-Raf mRNA expression levels. This finding may reveal how a shift away from survival can be modulated.

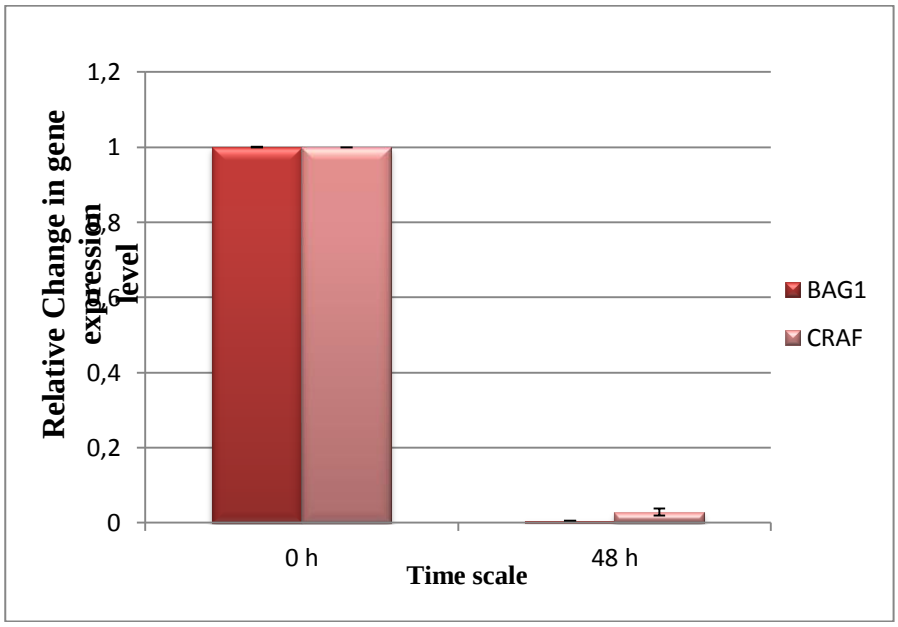


Figure 3.6: qPCR analysis of C-RAF gene after BAG-1 siRNA treatment in MCF-7 cells (mean: 22, std: 0.01414, *p < 0.05, 0.01)

3.3.3 Effect of BAG1 siRNA treatment on AKT gene expression in MCF7 cell line

AKT1, AKT2, and AKT3 make up a family of serine/threonine kinases, often activated downstream of growth factor receptors and PI 3-Kinase. Each AKT family member contains an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain. They have putative roles in cell proliferation and survival, and AKT perturbations are thought to play an important role in tumorigenesis. AKT (also called Akt1)/ (PKB), plays a key role in apoptosis, and it is involved in cellular survival pathways, by inhibiting apoptotic processes.

AKT is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy and general tissue growth. Since it can block apoptosis, and thereby promote cell survival, AKT has been implicated as a major factor in many types of cancer. AKT could promote growth factor-mediated cell survival both directly and indirectly. BAD is a pro-apoptotic protein of the BCL-2 family. AKT could phosphorylate BAD on Ser136, which makes BAD dissociate from the BCL-2/BCL-X complex and loses the pro-apoptotic function. AKT could also activate NF-κB via regulating IκB kinase (IKK), thus result in transcription of pro-survival genes. When we analyze BAG1silencing effect on AKT gene expression level as a marker for cell survival, we observed a decrease in its expression level (mean: 0.5535, std: 0.546, $p>0.05$) (Figure 3.7), so we can hypothesize that with a decrease in BAG1 level, cell promptly decreases AKT levels to escape from survival and direct the cell toward apoptosis leading to elevated percentage of cell death.

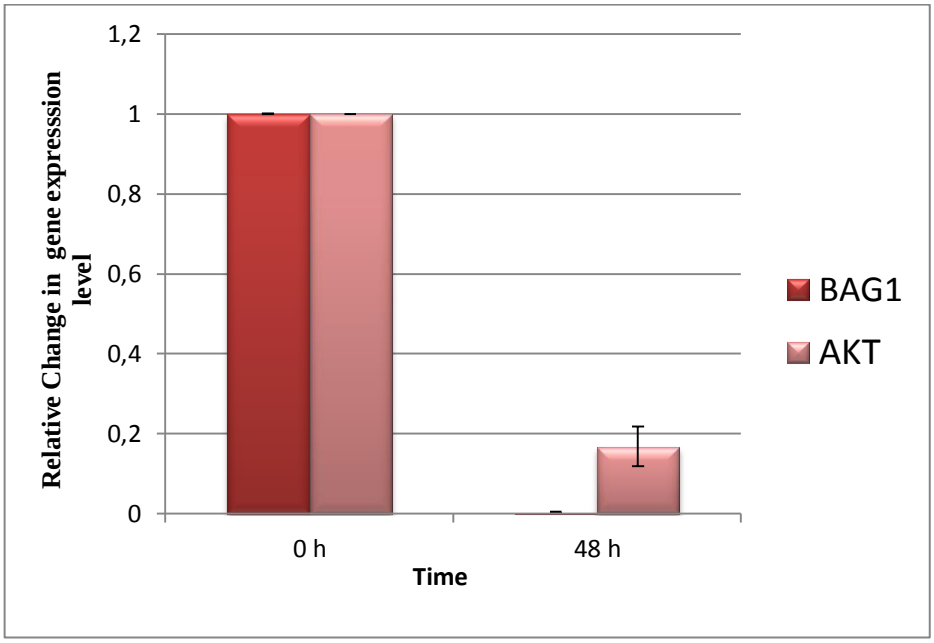


Figure3.7: qPCR analysis of AKT gene after BAG-1 siRNA treatment in MCF-cells (mean: 29.01, std: 0.07071, $p > 0.05$, 0.05)

3.3.4: Effect of BAG1 siRNA treatment on BCL2 gene expression in MCF7 cell line

When BAG1 was silenced, BCL2 transcript levels were sharply reduced (Figure 3.8). As known, BAG1 functions by directly influencing apoptosis of breast cancer cells in

a BCL2 dependent manner. BCL2 is an anti-apoptotic protein and its mRNA level decrease points to a direction away from survival in the cancerous cells.

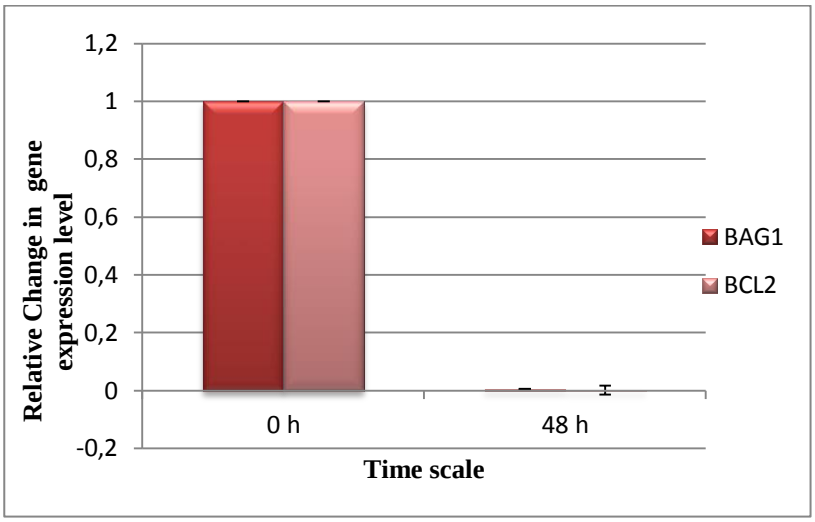


Figure 3.8: qPCR analysis of BCL2 gene after siRNA treatment (mean: 22.86, std: 0.02121, *p < 0.05, 0.015)

3.3.5 Effect of BAG1 siRNA treatment on BAX gene expression in MCF7 cell line.

When BAG1 silencing was applied to MCF7 cells, BAX transcript levels were elevated (Figure 3.9). Since BAX, which is a protein of the BCL2 gene family, promotes apoptosis by competing with BCL2, it is expected to observe an enhancement in the BAX levels when a pro-survival gene was knocked-down. The BAX gene was the first identified pro-apoptotic member of the BCL2 protein family. BCL2 family members share one or more of the four characteristic domains of homology entitled the BCL2 homology (BH) domains (named BH1, BH2, BH3 and BH4), and can form hetero- or homodimers. BCL2 proteins act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. In healthy mammalian cells, the majority of BAX is found in the cytosol, but upon initiation of apoptotic signaling, Bax undergoes a conformational shift. Upon induction of apoptosis, BAX becomes organelle membrane-associated, and in particular, mitochondrial membrane associated. BAX is believed to interact with, and induce the opening of the mitochondrial voltage-dependent anion channel, VDAC. Alternatively, growing evidence also suggests that activated BAX and/or Bak form an oligomeric pore, MAC, in the outer membrane, resulting in the release of cytochrome c and other pro-apoptotic factors from mitochondria and leading finally

to the activation of caspases. The expression of BAX is upregulated by the tumor suppressor protein p53, and BAX has been shown to be involved in p53-mediated apoptosis. The p53 protein is a transcription factor that, when activated as part of the cell's response to stress, regulates many downstream target genes, including BAX. Wild-type p53 has been demonstrated to upregulate the transcription of a chimeric reporter plasmid utilizing the consensus promoter sequence of BAX approximately 50-fold over mutant p53. Thus it is likely that p53 promotes BAX's apoptotic faculties in vivo as a primary transcription factor. However, p53 also has a transcription-independent role in apoptosis. In particular, p53 interacts with Bax, promoting its activation as well as its insertion into the mitochondrial membrane. BAX/BCL2 protein level of a cell is indicative of the cells apoptotic situation. From a parallel study (unpublished data), we determined that BAX/BCL2 ratio is dramatically increased in 48 h BAG1 siRNA transfected MCF-7 cells. mRNA levels and protein levels revealed similar results. Therefore, we think that with BAG1 silencing cells are directed away from survival.

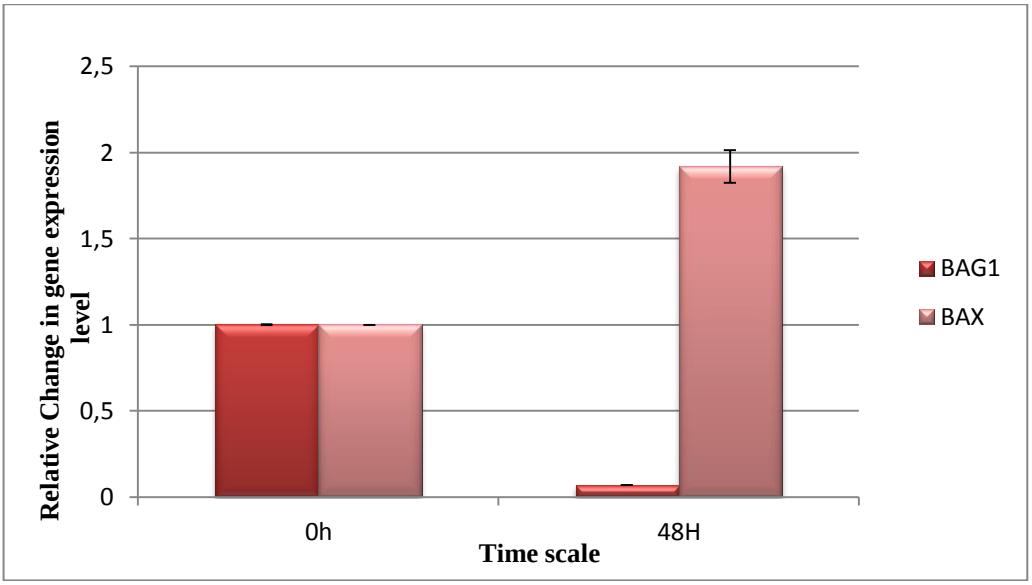


Figure 3.9. qPCR analysis of BAX gene after siRNA treatment (mean: 13.26, std: 0.1344, p>0.05, 0.095)

3.3.6 Effect of BAG1 siRNA treatment on BAD gene expression in MCF7 cell line

When we analyzed BAD transcript levels, we observed a dramatic enhancement with BAG1 silencing (mean: 4.094, std: 1.726, p>0.05) (Figure 3.10). Since the error between our measurements is big, the result is not obtained as significant; more

experiments should be performed to lower the error. Overall, though, the trend in enhancement of BAD transcript is consistent. The BCL2-associated death promoter (BAD) protein is a pro-apoptotic member of the BCL2 gene family which is involved in initiating apoptosis. BAD is a member of the BH3-only family. After activation, it is able to form a heterodimer with anti-apoptotic proteins and prevent them from stopping apoptosis. BADs phosphorylation status determines its activation or deactivation: Dephosphorylated BAD forms a heterodimer with BCL2 and BCLXL, inactivating them and thus allowing Bax/Bak-triggered apoptosis. When BAD is phosphorylated by Akt/protein kinase B (triggered by PIP3), it forms the BAD-(14-3-3)protein homodimer. This leaves BCL2 free to inhibit Bax-triggered apoptosis. BAD phosphorylation is thus anti-apoptotic, and BAD dephosphorylation (e.g., by Ca2+-stimulated Calcineurin) is pro-apoptotic. In a parallel study, when we checked the phosphorylation status of BAD in BAG1 overexpressed and silenced MCF-7 cells, we observed that BAD gets phosphorylated and dephosphorylated from residue 136, respectively. These results with the mRNA results revealed that pro-survival effects of BAG1 is suppressed under its silenced environment. BAG1 with our previous studies is partly affecting survival through varying the phosphorylation status of BAD.

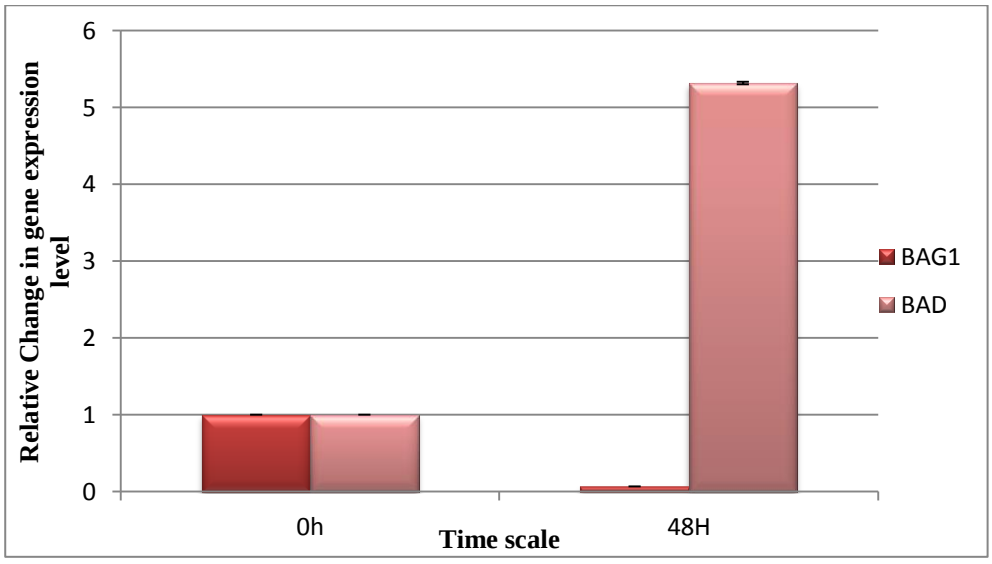


Figure 3.10: qPCR analysis of BAD gene after siRNA treatment (mean: 16, std: 0.02121, *p<0.05, 0.015)

3.3.5 Effect of BAG1 siRNA treatment on 14-3-3 gene expression in MCF7 cell line

To study the effect of BAG1 on survival through BAD and 14-3-3 route, we analyzed 14-3-3 transcript levels. When BAG1 was silenced, we observed a decrease in the transcript levels of 14-3-3 (Mean:-, std: 0.3536, $p>0.05$) (Figure 3.11). Statistical analysis results are not acceptable, but when we compare the trend of change in 14-3-3 levels, we observe in all the measurements a decrease upon BAG1 silencing. Further experiments should be performed to obtain consistent results.

14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells. There are at least seven distinct 14-3-3 genes in vertebrates: alpha/beta, epsilon, eta, gamma, theta, sigma, and zeta/delta. 14-3-3 proteins have the ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases and transmembrane receptors. 14-3-3 proteins act as key regulators of intracellular signal transduction through their ability to bind specific motifs containing phosphorylated serine or threonine residues.

Since 14-3-3 proteins have diverse functions, BAG1 level alterations can affect 14-3-3 levels differently. One possible effect would be through the mentioned survival path. Further studies are necessary to delineate the entire network of communications between BAG1 and 14-3-3.

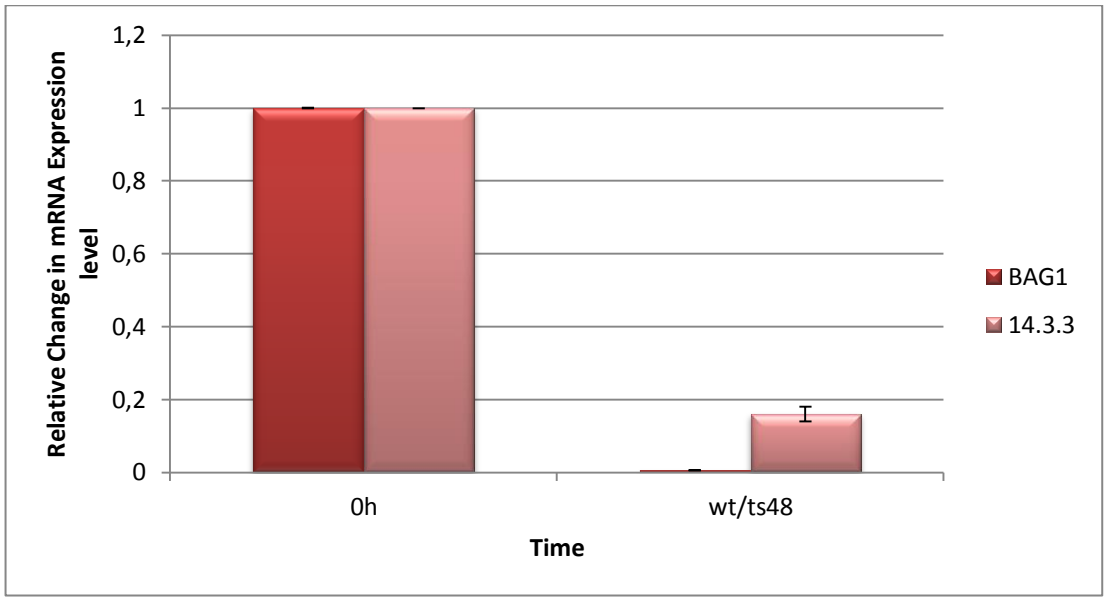


Figure 3.11: qPCR analysis of 14.3.3 gene after siRNA treatment (Mean:20.02, std: 0.03464, *$p<0.05$, 0.02)

BID is a member of the BCL2 family of proteins that regulates outer mitochondrial membrane permeability. BID is a proapoptotic member that causes cytochrome c to be released from the mitochondrial intermembrane space into the cytosol. In healthy cells, BID is cytosolic. In response to Fas Ligand or TNF-alpha, BID is cleaved by Caspase-8 and then relocates to the mitochondrial outer membrane.

3.4 Disccussion

Although some inconsistencies have been reported, there appear to be broad agreement that BAG1 is overexpressed in breast and non-small cell lung cancer and that this can correlate with clinical parameters and improved patient outcome. Studies have focused on breast cancer, non-small cell lung cancer and squamos cell carcinoma of the head and neck, and there are indications that BAG1 expression may also be altered in glioblastoma, cervical cancer and hematopoietic-malignancies. Further work, including prospective trials, are required to confirm the exciting possibility that BAG1 expression might be used as a prognostic marker in early breast cancer (Turner et al., 2001). Larger prospective studies should be more representive of the spectrum of breast cancer as a whole and have increased power to detect independent prognostic predictors in multivariate analysis,in particular in the presence of possible confounding associations such as tumor grade and Era status. As discussed by Turner, 2001, it is not immediately apparent why an antiapoptotic protein is associated with good prognosis. Multiple alteration contribute to carcinogenesis, and tumors that counter apoptosis by overexpressing BCL2 and BAG1 might represent one class of tumors. Other tumors may have disabled apoptotic response through accumulation of distinct changes, which might have more profound effects on cell death sensitivity or additionally affect proliferative pathway resulting in more aggressive tumour growth. It is also likely that the key targets of BAG1 will differ between cell types. A major factor that complicates the interpretation of immunohistochemical analyses stems from the fact that there are multiple BAG1 isoform that can be functionaly distinct. Thus, we should not expect a simple pattern of changes in BAG1 expression in all cancer types due to differences in key BAG1 targets in different cell types. Despite the interesting and encouraging results obtained from studies of BAG1 in breast cancer, the ability of

BAG1 overexpression to promote tumour formation in transgenic animals is yet to be tested. Such studies may produce compelling evidence that BAG1 plays a casual role in cancer development.

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APPENDICES

APPENDIX A: **Laboratory Equipment**

APPENDIX B: **Kits & Chemicals**

APPENDIX C: **Buffers & Media**

APPENDIX D: **Melting curves of our target gene amplification RT-PCRs**

APPENDIX E: **Table of statistical analysis results obtained from qRT-PCR**

APPENDIX A

Laboratory Equipment

Micropipettes	Eppendorf Research 2 μl 10 μl 20 μl 100 μl 200 μl 1000 μl
Pipettes	Serological Pipettes 2 ml, 5 ml, 10 ml, 25 ml
Centrifuge	Hettich ROTINA 420 Tabletop Centrifuge
Vortex	IKA MS3 Basic & Yellowline TTS2
Laminar Flow	Thermo Scientific HERAsafe Cabinets Class II
Incubator	Thermo Scientific Heraeus Function Line
Refrigerators	Liebherr $+4^0$C, FRYKA -30^0C, Kirsch -30^0C, New Brunswick Scientific -80^0C
PCR Thermocyclers	Bio Rad DNA Engine Dyad Peltier Thermal Cycler Eppendorf Mastercycler Thermal Cycler
Spectrophotometer	Thermo Scientific Nanodrop ND 1000 V 3.3 + Software
Cell Counter	Hemocytometer
qRT-PCR System	Roche LightCycler 480 Real-Time PCR System Software + geNorm algorithm data calculation
Microscope	Axiovert 25 Zeiss + AxioCam Icc 1 + Software

APPENDIX B

Kits & Chemicals

Disinfectants	Bacillol AF - Cellculture
	Applichem RNase-ExitusPlus
Ethanol	Carl Roth 70%, 99.9% (RNA purpose)
Trypsine	PAA
PBS	PAA Dulbecco's PBS (1x) without Ca & Mg
β-mercaptoethanol	Applichem β-mercaptoethanol (RNA purpose)
DMEM	PAA DMEM High Glucose (4,5 g/L) with L-Glutamin
FCS	Invitrogen
RNA Isolation	Roche high pure RNA isolation Kit
RT Reaction	Roche Transcriptor High Fidelity cDNA Synthesis Kit
qRT-PCR	Roche LightCycler 480 Probes Master (2 Conc.)
Primers	Sigma-Aldrich

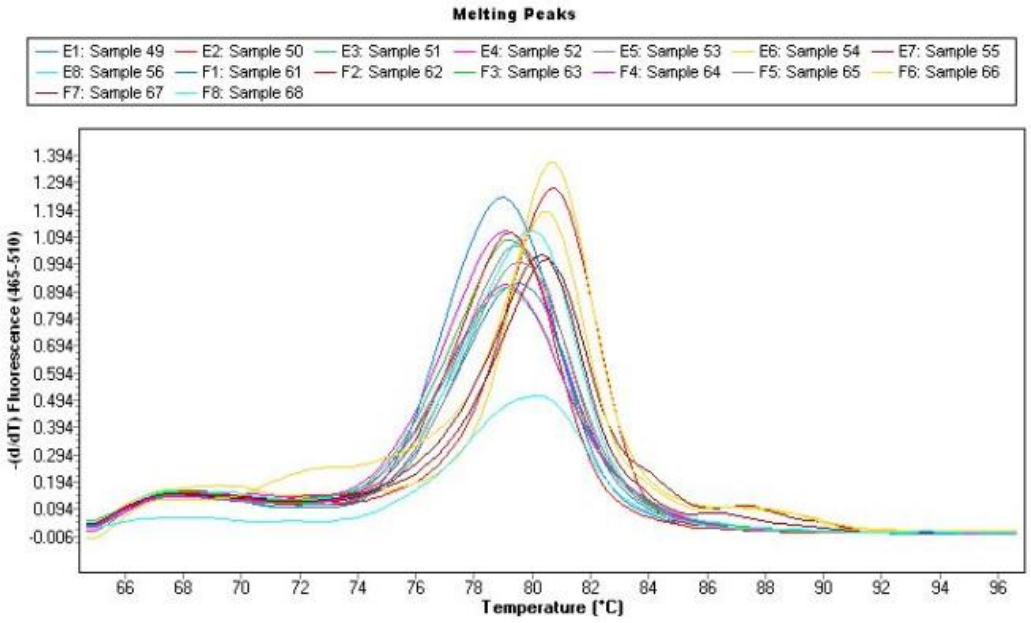
APPENDIX C

Buffers & Media

Growth Medium	500 ml DMEM HighGlucose (4,5 g/L) with L-Glutamine
	50 ml FCS
	5 ml 100x Penicillin/Streptomycin
Trypsine Solution	Trypsin/EDTA (0,025%/0,01%)

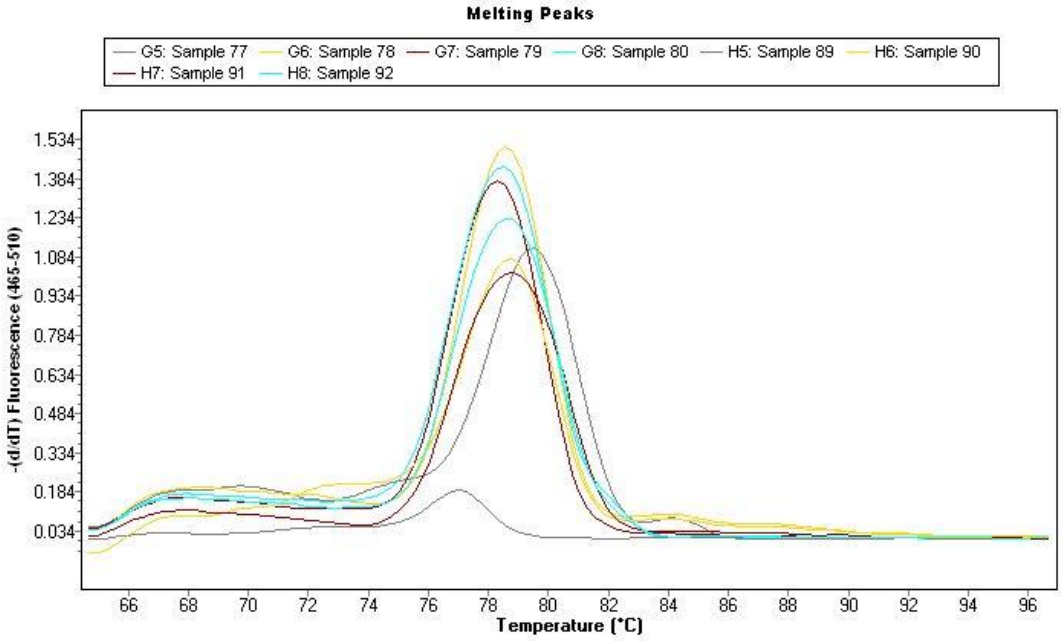
APPENDIX D

Melt Curve Genotyping for B_RAF (Melt Curve Genotyping)



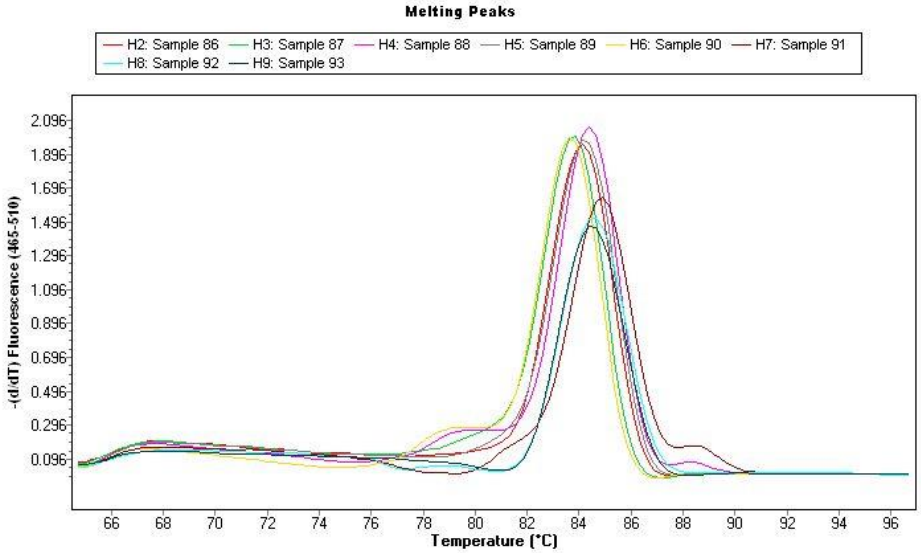
E1	BRAF wt 1/1	F1	BRAF wt 1/1
E2	BRAF wt 1/10	F2	BRAF wt 1/10
E3	BRAF wt 1/100	F3	BRAF wt 1/100
E4	BRAF WT 1/1000	F4	BRAF WT 1/1000
E5	BRAF TS48 1/1	F5	BRAF TS48 1/1
E6	BRAF TS48 1/10	F6	BRAF TS48 1/10
E7	BRAF TS48 1/100	F7	BRAF TS48 1/100
E8	BRAF TS48 1/1000	F8	BRAF TS48 1/1000

Melt Curve Genotyping for C_RAF (Melt Curve Genotyping)



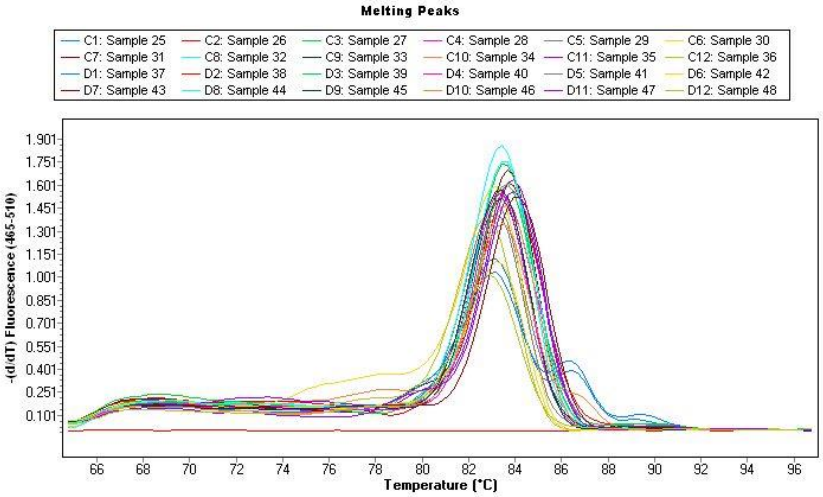
G5	CRAF wt 1/1
G6	CRAF wt 1/10
G7	CRAF wt 1/100
G8	CRAF WT 1/1000
H5	CRAF TS48 1/1
H6	CRAF TS48 1/10
H7	CRAF TS48 1/100
H8	CRAF TS48 1/1000

Melt Curve Genotyping for GAPDH (Melt Curve Genotyping)



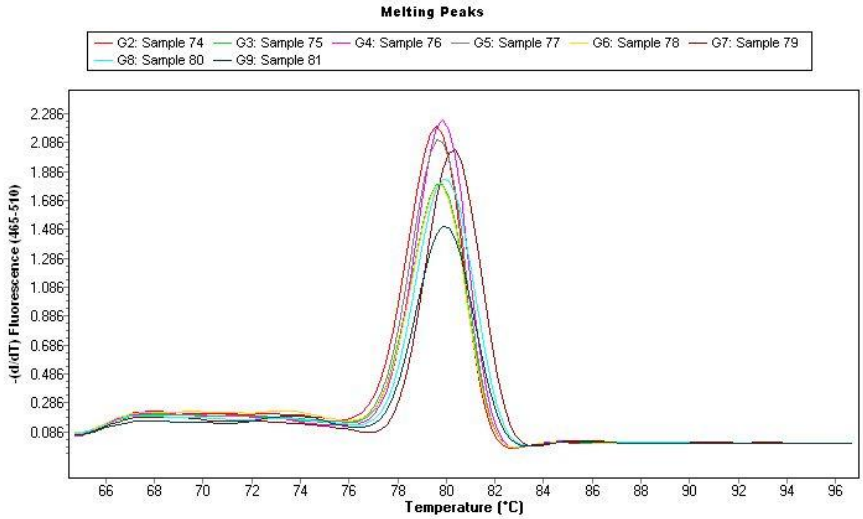
H2	GAPDH wt 1/1
H3	GAPDH wt 1/10
H4	GAPDH wt 1/100
H5	GAPDH WT 1/1000
H6	GAPDH TS48 1/1
H7	GAPDH TS48 1/10
H8	GAPDH TS48 1/100
H9	GAPDH TS48 1/1000

Melt Curve Genotyping for BCL2 (Melt Curve Genotyping)



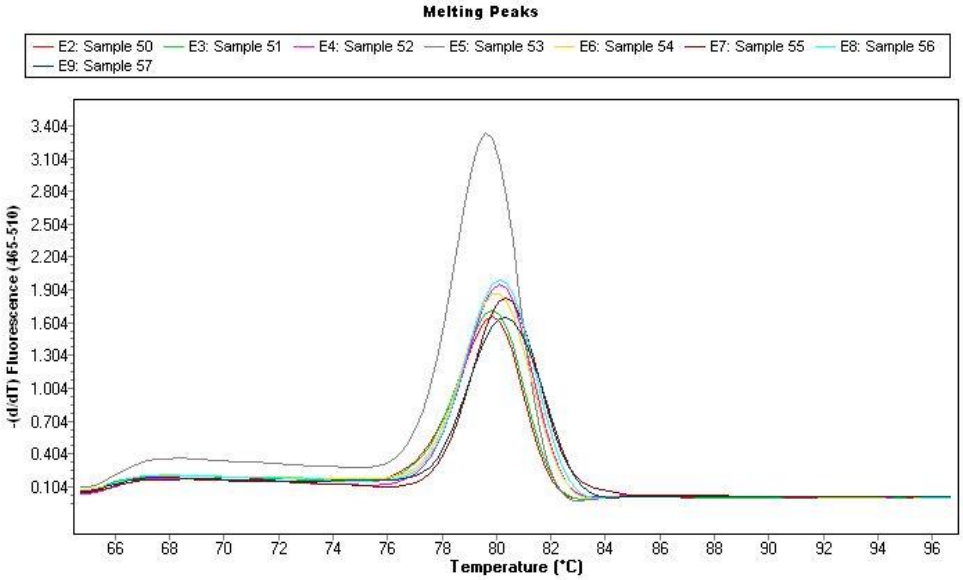
C1	BCL2 wt 1/1	D1	BCL2 wt 1/1
C2	BCL2 wt 1/10	D2	BCL2 wt 1/10
C3	BCL2 wt 1/100	D3	BCL2 wt 1/100
C4	BCL2 WT 1/1000	D4	BCL2 WT 1/1000
C5	BCL2 WT 1/10.000	D5	BCL2 WT 1/10.000
C6	BCL2 TS48 1/1	D6	BCL2 TS48 1/1
C7	BCL2 TS48 1/10	D7	BCL2 TS48 1/10
C8	BCL2 TS48 1/100	D8	BCL2 TS48 1/100
C9	BCL2 TS48 1/1000	D9	BCL2 TS48 1/1000
C10	BCL2 TS 1/10.000	D10	BCL2 TS 1/10.000
C11	Neg CONTROL	D11	Neg CONTROL

Melt Curve Genotyping for BAG1 (Melt Curve Genotyping)



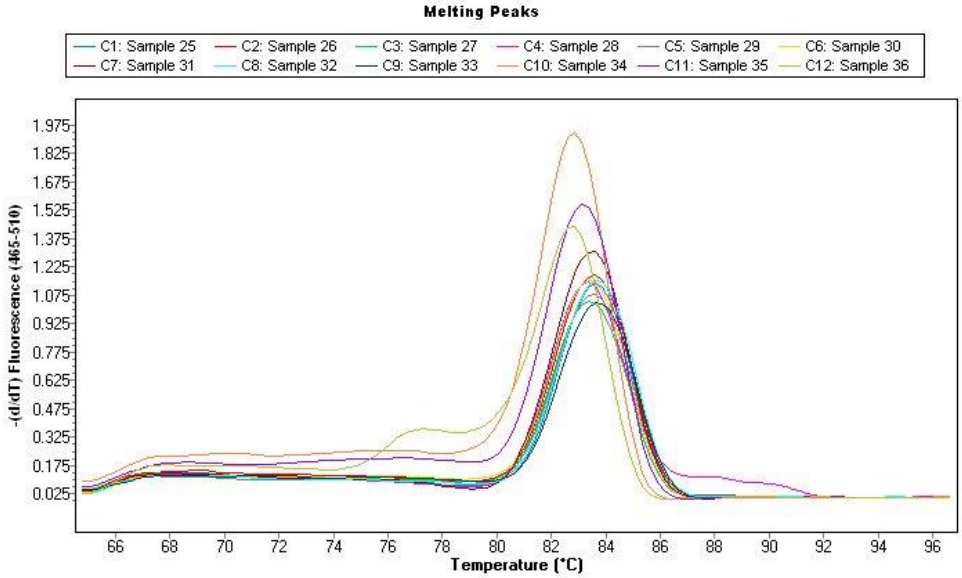
G2	BAG1 wt 1/1
G3	BAG1 wt 1/10
G4	BAG1 wt 1/100
G5	BAG1 wt 1/1000
G6	BAG1 TS48 1/1
G7	BAG1 TS48 1/10
G8	BAG1 TS48 1/100
G9	BAG1 TS48 1/1000

Melt Curve Genotyping for 14,3,3 (Melt Curve Genotyping)



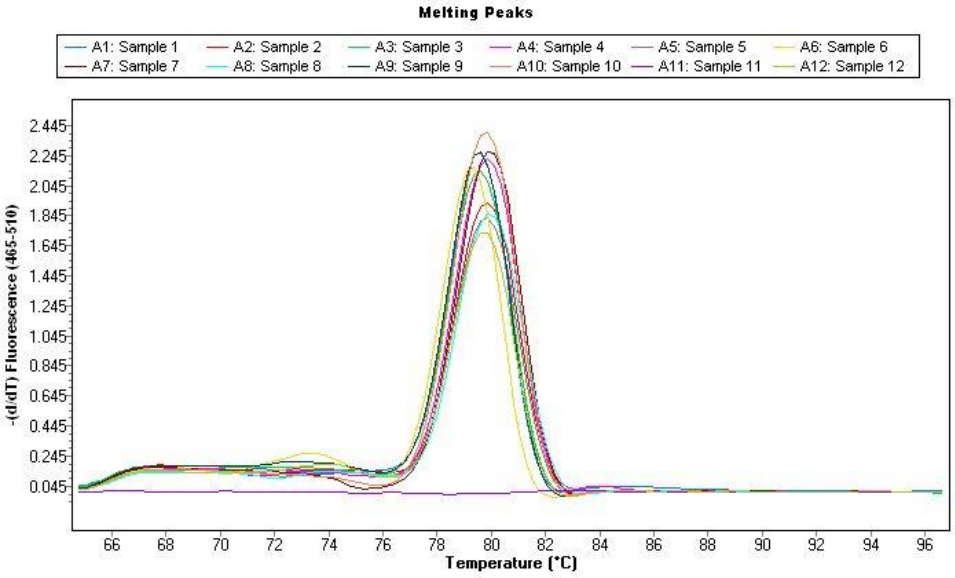
E2	14,3,3 wt 1/1
E3	14,3,3 wt 1/10
E4	14,3,3 wt 1/100
E5	14,3,3 wt 1/1000
E6	14,3,3 TS48 1/1
E7	14,3,3 TS48 1/10
E8	14,3,3 TS48 1/100
E9	14,3,3 TS48 1/1000

Melt Curve Genotyping for AKT (Melt Curve Genotyping)



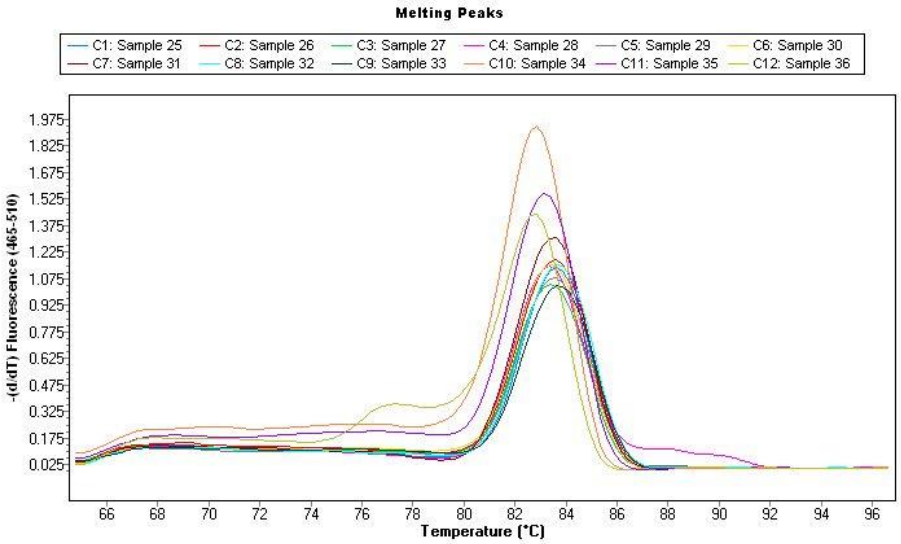
C1	AKT wt 1/1
C2	AKT wt 1/10
C3	AKT wt 1/100
C4	AKT WT 1/1000
C5	AKT WT 1/10.000
C6	AKT TS48 1/1
C7	AKT TS48 1/10
C8	AKT TS48 1/100
C9	AKT TS48 1/1000
C10	AKT TS 1/10.000
C11	
C12	Wrong

Melt Curve Genotyping for BAD (Melt Curve Genotyping)



A1	BAD wt 1/1
A2	BAD wt 1/10
A3	BAD wt 1/100
A4	BAD WT 1/1000
A5	BAD WT 1/10.000
A6	BAD TS48 1/1
A7	BAD TS48 1/10
A8	BAD TS48 1/100
A9	BAD TS48 1/1000
A10	BAD TS 1/10.000
A11	
A12	NEGATIVE CONTROL

Melt Curve Genotyping for BAX (Melt Curve Genotyping)



C1	BAX wt 1/1
C2	BAX wt 1/10
C3	BAX wt 1/100
C4	BAX WT 1/1000
C5	BAX WT 1/10.000
C6	BAX TS48 1/1
C7	BAX TS48 1/10
C8	BAX TS48 1/100
C9	BAX TS48 1/1000
C10	BAX TS 1/10.000

APPENDIX E

Table: Statistical analysis results obtained from qRT-PCR

Genes				**P value**
BAG1	Mean		0.004715	
	Std. Deviation		0.001477	
	Std. Error of Mean		0.001045	P value
B-Raf	Mean		20.96	
	Std. Deviation		0.3535	
	Std. Error of Mean		0.025	P value
C-Raf	Mean		22	
	Std. Deviation		0.01414	
	Std. Error of Mean		0.01	P value
AKT	Mean		29.01	
	Std. Deviation		0.7071	
	Std. Error of Mean		0.05	P value
BCL2	Mean		22.86	
	Std. Deviation		0.02121	
	Std. Error of Mean		0.015	P value
BAX	Mean		13.26	
	Std. Deviation		0.1344	
	Std. Error of Mean		0.095	P value
BAD	Mean		16	
	Std. Deviation		0.02121	
	Std. Error of Mean		0.015	P value
14-3-3	Mean		20.02	
	Std. Deviation		0.03464	
	Std. Error of Mean		0.02	P value

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