**T.C.**

**BIRUNI UNIVERSITY**

**INSTITUTE OF GRADUATE EDUCATION**

**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

**MOLECULAR AND MEDICAL GENETICS GRADUATE PROGRAM**

# TEMOZOLOMIDE RESISTANCE IN GLIOBLASTOMA; EFFECT OF CALCIUM-BINDING PROTEIN S100P

**Submitted by:**
**Fatma Ezzahara Khaled Gadur**

**Submitted to:**
**Asst. Prof. Dr. Elif Sibel Aslan**

**June, 2023**

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## DECLARATION

I hereby declare that the project work entitled “Temozolomide Resistance in Glioblastoma; Effect of Calcium-Binding Protein S100P” submitted to the Biruni university, is a record of an original work done by me under the guidance of Asst. Prof. Dr. Elif Sibel Aslan, Head Of Molecular biology & Genetics Department, Biruni university, and this project work is submitted in the fulfilment of the requirements for the award of Molecular and Medical Genetics Master degree. The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

## ACKNOWLEDGMENT

Completing this Thesis Writing entitled “Temozolomide Resistance in Glioblastoma; Effect of Calcium-Binding Protein S100P”- last requirement for the Master of Molecular and Medical Genetics is truly a marathon, and the researcher would not have been able to complete this journey without the aid and support of countless people who in one way or another contributed and extended their valuable assistance. The researcher would like to express his heartfelt gratitude, appreciation and thanks to the following individuals for reviewing parts of the manuscript, to Asst. Prof. Dr. Elif Sibel Aslan Head Of Molecular biology & Genetics Department, thesis adviser and statistician, for her continuous support, patience, motivation, valuable comments, suggestions and her immense knowledge and advises to make the research meaningful, and to Sajjad Eslamkhah who provided encouraging and constructive feedbacks and valuable comments and suggestions in developing the research and for his guidance and encouragement to finish the study.

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## Abbreviations

| | |
|---|---|
| **EGFR** | Epidermal growth factor receptor |
| **BBB** | Blood-brain barrier |
| **CNS.** | Central nervous system |
| **GBM** | Glioblastoma |
| **TME** | Tumor microenvironment |
| **CBTRUS** | Central Brain Tumour Registry of the United States |
| **CT** | Computed Tomography |
| **MRI** | Magnetic resonance imaging |
| **MRS** | Magnetic resonance spectroscopy |
| **WHO** | Word Health Organisation |
| **MGMT** | O6-methylguanine-DNA methyltransferase |
| **TERT** | Telomerase reverse transcriptase |
| **H3F3A** | H3 histone, family 3A |
| **FGFR** | Fibroblast growth factor receptors |
| **NTRK** | Neurotrophic tropomyosin kinase receptors |
| **GFAP** | Glial fibrillary acidic protein |
| **ANC** | Neutrophils Count |
| **MTIC** | 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide |
| **AIC** | 5-aminoimidazole-4-carboxamide |
| **MGMT** | Suicide methylguanine-DNA methyltransferase |
| **MMR** | DNA mismatch repair |
| **TMZ** | Temozolomide. |
| **EF-hand** | Helix-loop-helix |
| **TEM** | Transendothelial migration |
| **ICC** | Intrahepatic cholangiocarcinomas |
| **GEO** | Gene Expression Omnibus |
| **DEGs** | Differentially expressed genes |
| **FBS** | Fetal bovine serum |
| **DMSO** | Dimethyl sulfoxide |
| DMEM | Dulbeccos Modified Eagle Medium |
| qRT-PCR | Real-time polymerase chain reaction |
| **Ct** | Threshold cycle |
| **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |

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## I. Abstract

Glioblastoma multiforme (WHO grade IV) is a devastating and aggressive primary brain tumor, characterized by poor prognosis and lack of efficient standard-of-care treatments. The vast majority of patients receiving radiotherapy along with Temozolomide chemotherapy, tend to develop Temozolomide resistance, hence resulting in tumor recurrence. Regardless of the advanced Glioblastoma treatments, Temozolomide remains the backbone of high-grade glioblastoma treatment. The poor prognosis and chemoresistance of glioblastoma raise the necessity to further-develop and investigate in order to identify novel molecular targets involved with chemoresistance in glioblastoma. The Calcium-Binding Protein S100P is involved with Chemoresistance, metastasis, as well as poor clinical outcomes in many malignancies. This research aims to shed light on the role played by Calcium-Binding Protein (S100P) in Temozolomide resistance in glioblastoma.


**Keywords**: Brain Cancer, Glioblastoma, Temozolomide, Chemoresistance, Calcium-Binding Protein S100P.


## I. Özet

Glioblastoma multiforme (WHO derece IV), kötü prognoz ve etkili standart bakım tedavilerinin olmaması ile karakterize, yıkıcı ve agresif bir birincil beyin tümörüdür. Temozolomid kemoterapisi ile birlikte radyoterapi alan hastaların büyük çoğunluğu Temozolomid direnci geliştirme eğilimindedir ve bu nedenle tümör nüksüne neden olur. Gelişmiş Glioblastoma tedavilerinden bağımsız olarak Temozolomide, yüksek dereceli glioblastoma tedavisinin bel kemiği olmaya devam etmektedir. Glioblastomanın zayıf prognozu ve kemodirenci, glioblastomada kemodirenç ile ilgili yeni moleküler hedefleri belirlemek için daha fazla geliştirme ve araştırma gerekliliğini arttırır. Kalsiyum Bağlayıcı Protein S100P, birçok malignitede Kemorezistans, metastaz ve ayrıca kötü klinik sonuçlarla ilişkilidir. Bu araştırma, glioblastomada Temozolomid direncinde Kalsiyum Bağlayıcı Proteinin (S100P) oynadığı role ışık tutmayı amaçlamaktadır.

## II. Introduction

### II.1 Glioblastoma

#### II.1.1 Overview

Glioblastoma abbreviated as GBM and known as a grade IV astrocytoma, is a fast-proliferating and devastating brain cancer. Glioblastoma is the most frequently diagnosed form of brain cancer [1]. In most cases, glioblastoma tends to migrate to the nearby brain tissues rather than to the distant organs. Glioblastoma is a result of the abnormal proliferation of astrocytic cells which support nerve cells in healthy tissues. Glioblastoma usually occurs in the brain but in some cases, it also occurs in the spinal cord and originates in the brain de novo or develops from astrocytomas with a lower grade. In adults, glioblastoma usually arises in the cerebral hemispheres mainly in the brain's frontal and temporal lobes.

Glioblastoma is a malignant and aggressive tumor that leads to death in an average of six months or less if not treated. Once diagnosed, it is critical to receive immediate professional neurosurgical and neuro-oncological care to elongate the survival rate of patients [1].

#### II.1.2 Classifications of Glioblastomas

Glioblastomas are categorized into primary glioblastomas and secondary glioblastomas each of them develops via distinct genetic pathways in patients of distinct ages resulting in different clinical outcomes [2]. Primary glioblastomas represent 80% of glioblastomas, this subclass is observed in individuals with an average age of 62 years. On the other hand, secondary glioblastomas arise from oligodendroglioma or astrocytomas with a lower grade in individuals with an average age of 45 years. Secondary glioblastomas are often found in the brain's frontal lobe, experience a lower grade of necrosis, and maintain a more enhanced prognosis compared with primary glioblastomas [3].

The Cancer Genome Atlas genetically classified glioblastoma into three different subclasses: classical glioblastoma, proneural glioblastoma, and glioblastoma mesenchymal. Each subclass is distinguished by distinct genetic mutations inducing epidermal growth factor receptor (EGFR) activation, platelet-derived growth factor receptor alpha activation, and neurofibromin 1 deletions, respectively. These subclasses possess different prognostic values, the mesenchymal subclass exhibits the shortest survival rates, while the proneural subclass exhibits the longest survival rates [4]. The tumor microenvironment composition is associated with the molecular subclasses of glioblastoma. Mesenchymal subclass show increased expression levels of CD4+ T cells, macrophages, and neutrophils [5] which is also involved with higher glioblastoma grades [4,6].

### II.1.3 Treatment challenges

There are many factors contributing to the treatment deficiency in glioblastomas, the main factor is its localization in the brain given that the BBB (blood-brain barrier) possesses a physical barrier as well as a biochemical barrier to the central nervous system (CNS) for big molecules [7,8]. The second factor is the capability of glioblastomas to develop resistance to conventional therapy [9, 10,11,12], as well as its limited capacity for brain self-repair.

Another factor to take into consideration is malignant cell migration to adjacent brain tissue. The consequential neurotoxicity of glioblastoma treatment also plays a critical role in the treatment deficiency of GBMs. Furthermore, The impaired tumor blood supply acts as a factor via inhibiting effective drug delivery in glioblastoma. Moreover, Tumor-induced seizures and tumor capillary leakage also play a vital role, leading to an accumulation of fluid around the tumor, or what's known as peritumoral edema and intracranial hypertension. Inter-tumor and intra-tumor heterogeneity also play a role in treatment success rate since it presents a combination of diverse subclasses of glioblastoma [4] and stromal cells in the TME (tumor microenvironment) [13,14].

### II.1.4 Epodiomology

Glioblastomas have one of the shortest survival rates among all cancers [15] which is not surprising given that it is the most widespread, aggressive, and malignant brain and central nervous system tumors. CBTRUS-2013 Central Brain Tumour Registry of the United States- indicates that the average annual incidence rate of age-adjusted glioblastoma is 3.19 per 100,000 population, which is the most elevated incidence rate in the malignant CNS (central nervous system) and brain tumors [16]. Glioblastoma represents roughly 15% of all CNS cancers and approximately 45% of primary malignant brain cancers [16].

Glioblastomas are more reported in men than women, given that the incidence rate is 1.57 % higher in men than women [17]. Primary glioblastoma is more reported in men, while secondary glioblastoma is more reported in women [18]. Glioblastoma can occur at any age. Though it is more likely to occur in older adults given its median diagnosis age of 64 years and considered rare in younger ages and children. The age-related incidence rises at ages from 75 to 84 years and decreases after 85 years [3].

Even though GBM is the most frequent malignant brain cancer, the overall median survival rate is about 15 months, while the progression-free survival rate is only 6 months [1]. Yet, the survival rate is roughly 40% in the first year after diagnosis and in the second year after diagnosis, the survival rate is only 17%. The 5 years survival rate of GBM is 4.6% and it has not increased in the last 30 years [19,20,21].

### II.1.5 Etiology

In large proportions, no genetic or environmental factors have been identified. Glioblastomas are sporadic, just like most cancer types. Yet there are a couple of risk factors that led to glioblastoma in some cases. Research revealed an increased

prevalence (17%) of prior exposure to therapeutic radiotherapy in GMB patients [22]. Indicating that prior exposure to therapeutic radiation raises the risk of glioblastoma incidence. The latency between exposure to radiation and glioblastoma incidence ranges from a few years to many decades.

Research indicates a reduced glioblastoma incidence with allergies and atopic diseases [23]. Anti-inflammatory medications possess a protective impact on glioblastomas [24].
Research indicates no concrete proof of GMB associations with lifestyle such as alcohol consumption, smoking drug usage, or exposure to N- Nitroso compounds [25]. Research indicates that cell phones don't raise the risk of glioblastoma incidence. Yet, long-term usage of cell phones has not been fully examined and requires further research [26].

A set of hereditary cancer syndromes highly raise the incidences of glioblastomas, such as Li-Fraumeni syndrome and Lynch syndrome [27]. Other studies reported that the incidence of glioblastoma increases with reduced susceptibility to allergy, immune genes, immune factors, and specific single-nucleotide polymorphisms [24].

### II.1.6 Symptoms

The symptoms of glioblastoma include; persistent headaches followed by seizures, loss of appetite, focal neurological deficits, gradual speech difficulty, intracranial pressure like nausea, changes in the ability to think and learn, vomiting, double or blurred vision, changes in mood and personality, and new onset of seizures [3,26, 27].

### II.1.7 Diagnosis

State-of-the-art imaging technologies can accurately specify the site of brain cancer, such as computed tomography (CT or CAT scan) as well as MRI (magnetic resonance imaging). During surgery, intraoperative MRI can be used to guide tissue biopsies and tumor removal. For the examination of the tumor's chemical profile Magnetic resonance spectroscopy (MRS) is used [27].

#### II.1.7.1 Conventional MRI:

MRI stands for Magnetic resonance imaging which is the most significant imaging analysis for astrocytoma. MRI examination is performed twice firstly before the patient receives the IV contrast and then performed again after the patient receives the IV contrast. If the tumor displays the contrast and appears bright on images this indicates astrocytoma with a higher grade. Some imaging lines allow indications of brain infiltration, swelling, and tumor cellularity [27]. As shown in the figure below glioblastomas exhibit intense contrast enhancement as well as central necrosis, unlike Lower-grade gliomas that do not exhibit considerable contrast enhancement [27].

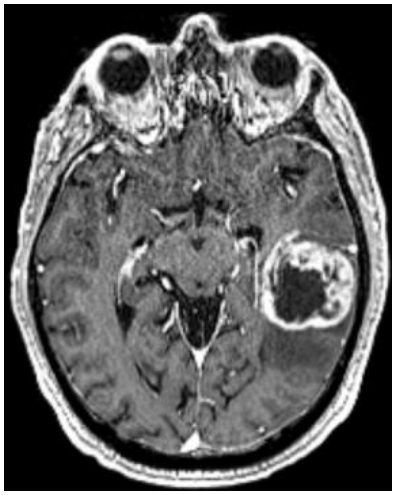

***Fig 1***. Post-gadolinium administration MRI spectroscopy [27].

**II.1.7.2 MRI spectroscopy (MRS):**

MRS is a non-invasive method used for tissue sampling, yet it is not as precise as a conventional biopsy. MRS is an imaging machine that is based on Magnetic resonance imaging, which gives an insight into the chemical and molecular composition of a given tumor and functions according to the fact that particular molecules exist abundantly in healthy brain tissues, while other molecules exist abundantly in tumor tissues such as choline. The results are illustrated in a diagram that shows the amount of each chemical in different brain areas. If the levels of NAA exceed choline, this indicates a healthy brain as shown in Figure 2 below. The contrary results increase the probability of a tumor [27].

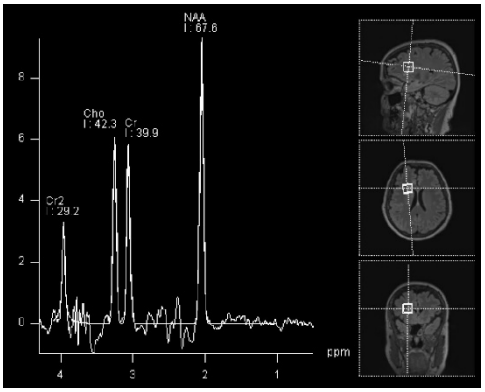


***Fig. 2*** MRI presents the levels of NAA exceeding choline indicating a healthy brain [27].

**II.1.7.3 fMRI (Functional Magnetic resonance imaging)**

Functional MRI is a valid practice used to discover the impacts of particular actions performed by the patient on the activity of specific brain parts. This practice is essential to determine the brain parts of the patient that would result in disabilities if impaired.

The parts that exhibit brain activity are illustrated in signals distinguished by red and yellow colors as shown in Figure 3 below [27].

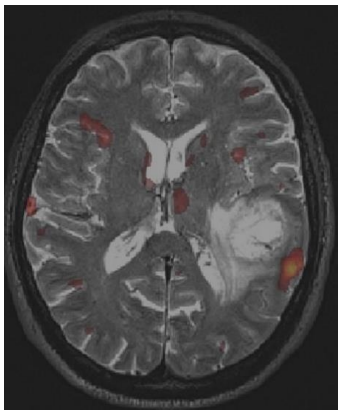

***Fig 3***. Functional MRI spectroscopy of a patient that was taken throughout object naming. [27]

For patients in which the tumor is found in functionally critical parts, such as the motor cortex, speech centers, or visual cortex, Functional MRI possesses a vital adjunct, especially regarding the planning of surgical intervention. The red and yellow signal exhibits considerable activity in the left temporoparietal area, in the anatomical part required for proper language production, and close to the glioblastoma site [27].

**II.1.8 Glioblastoma Grading**

Glioblastomas are categorized according to their morphology by WHO as grades 5 distinct. Grade I represents benign tumors, grade II represents moderately benign tumors, grade III represents lower-grade malignant tumors, and finally, grade IV includes glioblastoma multi-forme which is an aggressive malignant tumor.

As mentioned earlier, CT and MRI are used to scan for brain tumors, once a tumor is detected a biopsy is obtained and inspected by a neuropathologist. Tissue analysis is performed to designate the tumor's grade. The glioblastoma grades provide insight into the type and WHO tumor classification and the growth rate of the tumor. The table below shows the different grading of glioblastoma [27].

**Table 1**. Glioblastoma grading.

| Classification | Histologic Grades |
|---|---|
| Grade II | Cytologic atypia ( nuclear shape difference, size variation, and hyperchromasia |
| Grade III | Anaplasia and elevated mitotic activity (high cellularity) |
| Grade IV | Necrosis and microvascular proliferation. |

### II.1.9 Pathophysiology

Glioblastoma cells express multiple epigenetic and genetic modifications. these modifications are essential in recognizing, categorizing, and comprehending the specific behavior of a certain tumor as well as its resistance to a given treatment. Glioblastoma stem cells have key mutations in addition to triggering mutations. Primary glioblastoma tumors originate from neural stem cell precursors, while secondary glioblastomas originate from genetic mutations in mature neural cells such as astrocytes.

Genetic mutations express and suppress specific genes, resulting in cellular and extracellular matrix modifications that eventually lead to multiple biochemical forms. Thus, the name glioblastoma multiforme is derived from the degree of genotypic variations [28].

The table below shows a set of precise genetic modifications found in glioblastomas that can be useful for prognosis and therapy response prediction and could also be used to determine therapeutic targets. Next-generation sequencing provides molecular profiling and analysis of glioblastomas to enhance diagnostic precision, therapeutic target specification, and prognosis.

**Table 2**. Significant Molecular Modifications in Glioblastoma [27, 29, 30, 31,32]

| | |
|---|---|
| IDH mutation | Prognostic value, potential therapeutic target |
| MGMT methylation status | Prognostic value, predictive value for response to temozolomide |
| EGFR mutation | Diagnostic maker for glioblastoma, potential therapeutic target |
| TERT promoter mutation | Diagnostic maker for glioblastoma |
| Gain for 7p and loss of 10q | Diagnostic maker for glioblastoma |
| H3F3A | Diagnostic marker for a subset of gliomas (H3 K27M-mutant and H3 G34 mutant), therapeutic target |
| FGFR fusion | Therapeutic target |
| NTRK fusion | Therapeutic target |

### II.1.9 Histopathology

Glioblastoma is histopathologically characterized by necrosis, microvascular proliferation, pleomorphic cell composition, nuclear hyperchromasia, significant angiogenesis, and increased mitotic and cellular activity [33, 34]. Moreover, recently formed vessels comprise various Weibel-Palade bodies which do not exist in the

endothelial cells of healthy brains. Moreover, vessels might possess thrombi that lead to damage in endothelial cells and hence promote tumor proliferation [3].

The necrosis patterns differ in primary and secondary glioblastoma. In primary glioblastoma, the necrotic regions exist in the large necrotic areas in the center leading to lack in blood supply, and also exist in several small foci surrounded by pseud-palisading. While in secondary glioblastoma, necrotic regions only exist in several small foci surrounded by pseudo-palisading [26]. Astrocytomas possess GFAP (glial fibrillary acidic protein). Loss of its expression implies high malignancy and indicates undifferentiated tumor cells [3].

### II.1.10 Glioblastoma Treatment

Current glioblastoma treatment care includes maximal safe surgical resection ensuring the removal of the tumor accompanied by radiation and adjuvant chemotherapy. During the tumor resection surgery, the tumor is removed as much as possible without damaging the nearby healthy brain tissues required for healthy neurological activity. Nevertheless, glioblastomas are enclosed by a surrounding of tumor cells that migrate, penetrate, and invade nearby healthy tissue, hence it is difficult to resect the tumor completely. Yet tumor resection comes in quite handy with cells that show resistance to chemotherapy and/or radiotherapy and hence decrease intracranial pressure. Moreover, debulking the tumor during surgery helps increase the life span of patients and enhances their life quality.

In the majority of cases, a craniotomy is performed, in which the skull is opened to access the tumor location. This is often performed with computer-guided imaging and employing intra-operative mapping approaches to specify the sites of the language corte, motor, speech cortex, and sensory. Intraoperative mapping is performed during the operation on an awake patient and mapping the anatomy of their language function throughout the operation to determine which tumor parts can be removed without causing any disabilities

Post-surgery treatments include radiation therapy and chemotherapy, which can begin once the wound is healed. Radiation selectively destroys the remaining tumor cells. In standard external beam radiation therapy, several radiation fractions sessions are exposed to the tumor site and its surroundings to eliminate the migrating infiltrating tumor cells. Each session damages healthy and tumor tissues, with a time gap between sessions to only allow normal cells to repair their damage. This procedure is performed in 10 to 30 sessions depending on the case, frequently delivered once a day or five days a week. Radiation therapy results in enhanced results and more prolonged survival rates in comparison with surgery alone.

Radiosurgery is a therapy procedure that employs technological radiation delivery systems to concentrate radiation at the location of the tumor tissues while decreasing the radiation exposure to the nearby healthy brain tissues. Radiosurgery is applicable for cases with tumor recurrence usually utilizing further data based on MRS or PET scans. Patients receiving chemotherapy are given certain chemicals designed to destroy tumor tissues. Temozolomide is the current mainstay chemotherapy for glioblastoma. Daily Drug administration is usually performed during radiotherapy treatment and for six cycles post-radiotherapy sessions throughout the maintenance stage. Every cycle is 28 days long, in which temozolomide is administrated in the first 5 days of every cycle, followed by rest for 23 days.
Furthermore, the Tumor Treating Fields treatment is administrated throughout the maintenance stage, it involves exposure to alternating electrical fields, which help inhibit cancer cells from growing and dividing. Tumor treating fields treatment combined with chemotherapy enhanced the median overall survival of patients from 16 to 20.9 months. Last but not least, Lomustine chemotherapy and bevacizumab targeted therapy are both given to patients with progressed tumors [30, 27, 35,36, 37]

### II.2 Temozolomide Resistance

Temozolomide is an alkylating chemical utilized as a chemotherapy treatment in glioblastoma multiforme as well as refractory anaplastic astrocytoma. In 1999 temozolomide was FDA-approved as an oral capsule, while the intravenous injection was FDA-approved on February 27, 2009 [38]. The Chemical Formula of Temozolomide is (C6H6N6O2) and its chemical structure is shown in Figure 1 below.

Temozolomide is an imidazotetrazine pro-drug characterized by acidic pH stability yet meets spontaneous non-enzymatic hydrolysis at neutral or merely basic pH. The given characteristics of Temozolomide permit its oral and intravenous applications [38, 39,40, 41]. After initial hydrolysis, additional reactions release a significantly reactive methyl diazonium cation with the ability to methylate diverse residues on adenosine and guanine bases resulting in DNA lesions and eventually apoptosis [40, 41]. Regardless of the significant genetic heterogeneity, Glioblastoma usually possesses impaired DNA repair systems, resulting in alkylating agent sensitivity. Unfortunately -in the long term- glioblastoma tumors tend to develop resistance to alkylating agents [38, 42, 43, 44].

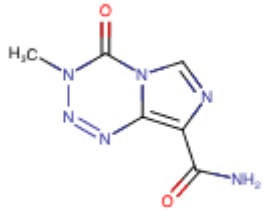


***Fig. 4*** Chemical Structure of Temozolomide.

Temozolomide treatment induces myelosuppression in which bone marrow activity declines leading to a decrease in red blood cells, platelets, and white blood cells. Myelosuppression is observed to be more intense in females and geriatric patients. Given the fact that Temozolomide treatment and myelosuppression are associated - prior to Temozolomide treatment- the platelet count should be ≥100 x 109/L, and the patient's ANC (neutrophils count) should be ≥1.5 x 109/L. ANC and platelet count should be monitored weekly throughout the treatment and until recovery [38, 44].
Patients receiving Temozolomide treatment are observed to develop myelodysplastic syndrome and myeloid leukemia as a secondary malignancy. Temozolomide patients are also susceptible to Pneumocystis pneumonia and severe hepatotoxicity. Moreover, experiments conducted on animals indicate that temozolomide causes severe embryo-fetal toxicity for both male and female patients, hence patients must follow a contraception routine for three months in males and six months in females after receiving temozolomide treatment [38].

Temozolomide is abbreviated as TMZ which is a small lipophilic alkylating agent with only 194 Da that falls under imidazotetrazine category. Imidazotetrazines are organic polycyclic compounds composing an imidazole ring linked to a tetrazine ring. Imidazole is a 5-membered ring made up of three carbons, and two nitrogens centered at the 1- and 3-positions. Tetrazine is made up of two carbons and four nitrogens and it is a 6-membered aromatic heterocycle [38, 41, 43, 44, 45].

Temozolomide is capable of crossing the BBB (blood-brain barrier) to act on CNS tumors. Once absorbed, Temozolomide spontaneously and non-enzymatically breaks down at physiological pH forming MTIC (5-(3-methyltriazen-1-yl) imidazole-4-carboxamide), and starts reacting with water forming AIC (5-aminoimidazole-4-carboxamide) and an extremely active methyl-diazonium cation. Glioblastoma and brain tumors in general retain more alkaline pH levels than normal tissues, favoring Temozolomide activation in tumor tissues [41,44].

The methyl-diazonium cation favors DNA methylation of guanine at the N7 and O6 position (N7-MeG 70%), (O6-MeG, 6%), adenine at the N3 position (N3-MeA, 9%). While N3-MeA and N7-MeG are quickly restored via the base-excision repair pathway and are not direct mediators of TMZ toxicity, lesions such as N3-MeA are fatal if do not go through repairing. On the other hand, repairing O6-MeG demands an enzyme called MGMT (suicide methylguanine-DNA methyltransferase), which repairs guanine by removing the methyl group. O6-MeG mispairs with thymine if not restored by MGMT leads to the activation of the MMR pathway (DNA mismatch repair) removing the thymine (not the O6-MeG), causing ineffective repair cycles and eventually breaking the DNA strand resulting in apoptosis [41, 44].

Since DNA mismatch repair pathway activation is essential for TMZ cytotoxicity, cells that harbor decreased or missing MGMT activity and functional MMR pathway are the most susceptible to TMZ administration. Temozolomide treatment failure in glioblastomas involves the upregulation of MGMT and the downregulation of MMR (TMZ resistance) [41, 43, 44].

Recent studies focused on the immunomodulatory impacts of TMZ and its associations with its myelosuppressive impacts indicated that lymphodepletion may improve the antitumor effect of cellular immunotherapy and enhance memory cell dynamics by modifying tumor-specific and tumor-tolerant cells. The diminished immunosuppressive Treg cells at the tumor site can potentially induce enhanced immunotherapy responses [46].

In terms of toxicity, myelosuppression is the major dose-limiting toxicity of temozolomide. Even though myelosuppression is likely to arise at any dosage, it is more severe at increased dosages [38]. Patients receiving higher dosages encountered adverse reactions, such as infection, prolonged and intense myelosuppression, and even death. Patients who received 2000 mg per day for five days encountered pyrexia, pancytopenia, and multiorgan failure, which eventually led to death. Hence complete blood count administration is crucial for patients with temozolomide overdose [38, 44]. Evidence revealed that combining TMZ chemotherapy with radiation resulted in a two-month increase in overall survival in glioblastoma [47].

### II.3 Calcium-Binding Protein P S100P

Ca2+-binding protein P -abbreviated as S100P- is a S100 family member. This family of proteins is composed of two EF-hand (helix-loop-helix) calcium-binding motifs, and are located in the nucleus and/or cytoplasm of a broad spectrum of cells and are associated with vast cellular activities including cell cycle progression and differentiation, regulation of calcium homeostasis, cell proliferation, cell invasion, apoptosis, protein phosphorylation, cell motility, cancer metastasis, autoimmunity, Chemotaxis, angiogenesis, regulation of transcriptional factors, cytoskeleton interactions, and inflammation [48]. There are more than 20 small dimeric calcium-binding proteins in the S100 family, hence it is considered the greatest group of the EF-hand superfamily [49].

The S100 genes family members exist as a cluster on chromosome 1q21; yet, S100P gene is localized at chromosome 4p16 as shown in Figure 3 below. S100P protein binds to Ca2+, Zn2+, and Mg2+ [50].


**Fig. 5** S100P location on Chromosome 4 - NC_000004.12

S100P protein was initially discovered in the placenta of humo sapiens [48] it is also found in other organs as shown in Figure 4 below which depicts an experiment in which RNA sequencing was performed on 95 human tissue samples from 27 distinct tissues to demonstrate the tissue-specificity of SP100[50].

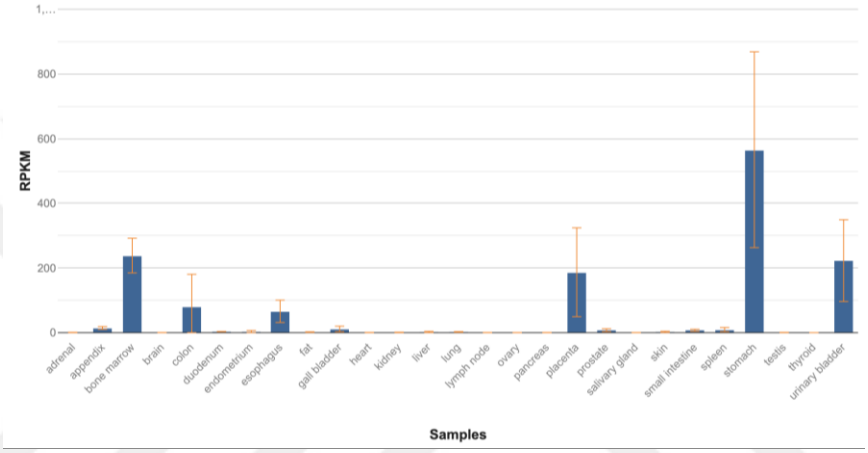


**Fig. 6**. Expression levels of SP100 in different cell types.

S100P possesses a distinct structural domain called EF-hand motif, which is found as intracellular or secreted homodimers or heterodimers. The EF-hand motif composition differs from one cellular context to another [51].

The roles played by S100P primarily depend on its regulations of or interactions with diverse molecules that carry out the regulation of extracellular matrix remodelings, and actin cytoskeleton dynamics such as Ezrin, myosin IIA, IQGAP1, cathepsin D, and phosphorylated cofilin [52,52,54,55]. S100P functions as a signaling molecule both, intracellularly and extracellularly [65, 57, 58]. Even though the mechanisms of S100P regulations have not been thoroughly reported in DNA, microarray reveals that S100P is upregulated by estradiol [59], progesterone [60], and HER2 overexpression [61].

S100P acts as an oncogene by activating RAGE which is a receptor for advanced glycation end product [62]. The extracellular ligand-binding domain of this receptor binds to various ligands such as S100P and triggers a downstream signaling pathway that stimulates cell proliferation, viability, as well as cell motility. studies reported that blocking the interactions between S100P and RAGE results in efficient inhibition of tumor growth [63].

Furthermore, the elevation in cellular calcium concentrations results in the formation of S100P dimers that act by binding and activating cytoplasmic proteins known as Ezrin [64]. The interactions between Ezrin and S100P dimers stimulate TEM (transendothelial migration) in patients with pancreatic cancer, lung cancer, and TNBC [65]. Additionally, S100P upregulates cyclin D1 and CDK2 which in response promoted cell proliferation in hepatocellular carcinoma [66]. The accumulation of S100P protein in tumor cells promotes the invasion of tumors by triggering angiogenesis [67]. S100P is predominantly found in the nucleus and cytoplasm of cells, yet it is found in the extracellular matrix as well, in both autocrine and paracrine manner [68].

Elevated plasma levels of S100P are highly involved with the deficient prognosis of MBC patients (metastatic breast cancer). Research indicates that radiation therapy of these patients results in a decrease in plasma levels of S100P [69].

S100P plays a vital role in drug responses in various types of cancer, either by promoting drug resistance [71, 71] or improving drug sensitivity [72]. In pancreatic cancer, the overexpression of S100P impacts 5-fluorouracil resistance [73], and also affects the irinotecan resistance in prostate cancer [74]. Moreover, the overexpression of S100P is correlated with the resistance to several drugs including cyclophosphamide, mitoxantrone, etoposide, and methotrexate in various cancer cell lines [75].

Studies also showed that S100P binds p53 and its ts negative regulator HDM2 which blocks p53-HDM2 complex binding resulting in an increase of the p53 level. The p53 induced by S100P impairs the activation of its transcriptional targets such as bax, hdm2, and p21WAF after DNA damage. S100P improves chemoresistance via binding and inactivating p53 [76]. On the other hand, a study conducted on patients with ovarian cancer cells revealed a
chemosensitization impact of S100P in different chemotherapeutic drugs such as carboplatin, 5-fluorouracil, paclitaxel, doxorubicin, and etoposide [77]. another study conducted on 8307 cell lines (colon cancer) indicates that S100P is involved with oxaliplatin sensitivity in cells that exhibit drug resistance [78]. These discoveries imply that both chemo-resistance and chemo-sensitivity can be fine-tuned by S100P in several cancers [79].

Studies associate S100P with increased cell motility and proliferation favoring the progression of diverse solid cancers [80, 81]. In addition, a study performed on benign rat mammary cell lines indicated that the overexpression of S100P stimulates metastasis [82]. Other studies associate S100P mechanistically with high cell migration and collective cell invasion [83,84]. S100P expression promotes cell motility and proliferation in vitro and carries out CRC metastasis in vivo [85].

The crystal structure of calcium-binding proteins S100P indicates that the homodimer S100P is made up o f95 amino acid monomer exhibiting a four α-helical structure and

the gene that codes for S100P is located on the human chromosome 4, at 4p16 [86, 87]. This specific chromosomal site has been linked with Huntington's disease [88], Wolf–Hirschhorn syndrome, Familial Wolfram syndrome [89, 90], Crohn's disease [91], and cervical cancer [92, 93].

In gastrointestinal cancers, S100P acts as a prognostic biomarker [94, 95]. Recent publications pursue enhancing the detection of oral cancer from salivary proteins, salivary RNA, salivary proteases, proteomic, and transcriptomic classes of several biomarkers including S100P. S100P transcripts were significantly increased in Oral squamous cell carcinoma [96]. In a recent study single-cell sequencing of RNA was performed on 144,878 samples from 14 pairs of iCCA tumors and normal liver tissues, the study revealed that S100P acts as a marker for iCCA perihilar large duct type (iCCAphl) and peripheral small duct type (iCCApps). S100P+ iCCAphl has considerably decreased levels of infiltrating CD4+ T cells, CD56+ NK cells, and elevated CCL18+ macrophages and PD1+CD8+ T cells in comparison with S100P- iCCApps [97].

S100P is overexpressed in mucin-secreting intrahepatic cholangiocarcinoma and is indicated to be a vital biomarker for perihilar large duct type cancer (iCCAphl) [98,99]. S100P is used as a non-invasive biomarker to identify non-metastatic pancreatic cancer [100]. S100P plays a noteworthy role in the tumorigenesis and immune microenvironment of pancreatic cancer suggesting that S100P presents a novel immune-related biomarker [101]. S100P has been previously reported as a metastasis and progression-related gene in ICC [102, 103]. S100P is a promising immunocytochemical marker [104].

S100P is upregulated in several types of cancer and plays a crucial role in the progression of tumors [105, 106, 107]**.** A recent study discovered that KRT17 and S100P genes were highly associated in nine intrahepatic cholangiocarcinomas (ICC) gene expression datasets, ICCs with S100P and KRT17 upregulation were involved with a poorer prognosis than those with downregulated S100P and KRT17 [108]. Survival analysis showed decreased survival for patients with upregulated S100P in HMECs cells [109]. In Lung Adenocarcinoma S100P was shown to be highly associated with the prognosis of LUAD patients. S100P drives TAMs (Tumor-associated macrophages) migration and M2 polarization in the immuno-suppressive tumor niche. Moreover, S100P drives the recruitment and polarization of TAMs in tumor tissues, which are crucial in the formation of an immunosuppressive tumor niche [110].

The upregulation of miR495 or S100P knockdown depressed the proliferation and invasion of pancreatic adenocarcinoma cells and promoted its apoptosis [111,112,113,114], S100P also plays a role in dysplastic leukoplakia and oral squamous cell carcinoma [115]. Moreover, elevated immuno-histochemical levels of S100P are correlated with significantly decreased survival rates of patients with breast [116, 117],

Hepatocellular [118,119] early stage non-small cell lung [120,121, 122, [123], Colon [124], Gallbladder Cancer [125, 126,127] and ovarian cancers [128,129] Breast cancer [130,131,132,133] prostate cancer [134], human acute myeloid leukemia [135], Colorectal Neoplasia [136,137,138]

Last but not least recent research indicates that S100P protein is a potent inducer of metastasis, and it is significantly correlated with decreased survival times in cancer. This study's findings pinpoint at least two S100P-dependent migration pathways, the first one is extracellular and the second one is intracellular, and indicate that the C-terminal lysine of S100P can be used as a target for the inhibition of several migration-inducing interactions of S100P proteins and S100P-based metastasis [139].

## III. Materials and Methods

### III.1. Dataset selection and data preprocessing

For this study, we selected the microarray gene expression data of U251 cells that were both sensitive and resistant to temozolomide. The dataset was obtained from the Gene Expression Omnibus (GEO) database, using the accession number GSE100736 (Available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100736). This dataset comprised a total of six samples, three samples representing temozolomide-sensitive cells and the other three samples representing temozolomide-resistant cells. To ensure the reliability and accuracy of the analysis, we performed data preprocessing using R version 4.2.3 and several relevant packages, including LIMMA [140], GEOquery [141], Biobase, and ggplot2 [142]. The data preprocessing steps involved background correction and normalization. Background correction was carried out using the NormalizeQuantiles function within the LIMMA package. This step helps eliminate systematic variations in the data caused by factors unrelated to the gene expression itself.

After background correction, normalization techniques were applied to ensure that the gene expression values were on a comparable scale. And for this purpose, the Normalize Quantiles function from the LIMMA package was used. By normalizing the data, we could minimize technical variations introduced during the experimental process. Next, we performed data filtering to remove probes with low expression values that could potentially introduce noise into the analysis. We retained only those probes that exhibited expression in at least 50% of the samples within each group (temozolomide-sensitive and temozolomide-resistant cells). This step ensured that the selected probes were representative of the gene expression patterns in each group.

To determine genes that were differentially expressed between temozolomide-sensitive and resistant U251 cells, we conducted differential expression analysis. This analysis was performed using the limma package in R, which employs a linear model approach to estimate the differential expression. The DEG analysis was based on the following

criteria: an absolute log fold-change (|logFC|) greater than or equal to 5 and an adjusted p-value less than 0.0001. These thresholds were chosen to prioritize genes that exhibited substantial changes in expression levels between the two cell groups while also ensuring statistical significance. By following these rigorous data preprocessing and analysis steps, we aimed to identify robust differentially expressed genes (DEGs) that could potentially be associated with temozolomide resistance in U251 cells.

### III.2. Co-expression network analysis

To gain a deeper understanding of the interrelationships among the differentially expressed genes (DEGs) pinpointed in our study, we conducted co-expression network analysis. This analysis was performed using Cytoscape version 3.9.1 [143] along with the GeneMANIA plugin [144]. The GeneMANIA plugin facilitated the construction of a co-expression network by integrating various types of functional interaction data, including co-expression, co-localization, and shared protein domains. This comprehensive approach allowed us to explore potential functional relationships among the DEGs.

To visualize the co-expression network, we employed the force-directed layout algorithm available in Cytoscape. This algorithm strategically positions nodes that exhibit strong connectivity in close proximity to one another. This visualization approach helps identify clusters or modules of genes that may share common functions or participate in similar biological processes. Furthermore, we utilized the NetworkAnalyzer plugin to evaluate the network's topological characteristics and identify the most interactive DEGs within the network. Various measures such as degree centrality and betweenness centrality were employed to assess the significance of individual nodes in the network. Degree centrality represents the number of connections a gene has with other genes, while betweenness centrality indicates the extent to which a gene serves as a bridge between different modules or clusters within the network.

The co-expression network, along with the identification of the most interactive DEGs, provides valuable insights into potential functional relationships and pathways that could be involved in glioblastoma. By analyzing the network, we can uncover key genes or modules that play crucial roles in the disease's molecular mechanisms. To further elucidate the biological significance of the identified genes, we recommend conducting functional annotation and pathway analysis. These additional analyses can shed light on the specific molecular functions, biological processes, and pathways in which the DEGs are involved. Such information could aid in unraveling the underlying mechanisms of glioblastoma and potentially screen for novel therapeutic targets for this devastating disease.

### III.3. Cell lines and culture

The U-251 human glioblastoma cell line, a widely used cell line for glioblastoma cancer research, was obtained commercially. The cells were cultured in RPMI-1640 media

supplemented with 1% antibiotics and 10% fetal bovine serum (FBS). This culture medium provided the necessary nutrients and growth factors to support cell growth and viability. The addition of antibiotics helped prevent contamination during cell culture. The cell culture was maintained in a humidified incubator with 5% carbon dioxide at a constant temperature of 37 degrees Celsius. This controlled environment mimicked physiological conditions suitable for the growth and survival of the U-251 cells.

For subculturing or passaging, the cells were detached from the culture flask using trypsinization. Trypsin, a proteolytic enzyme, facilitated the detachment of cells from the culture surface, allowing for their subsequent transfer to new culture vessels. The detached cells were then resuspended in a fresh medium for further growth and experimentation. To ensure long-term storage and preservation of the U-251 cells, cryopreservation techniques were employed. The cells were cryopreserved in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 10% dimethyl sulfoxide (DMSO). The addition of DMSO acted as a cryoprotectant, preventing damage to the cells during the freezing and thawing processes.

Cryopreservation allowed for the establishment of a cell bank for future experiments and maintained the genetic stability of the cell line. Throughout the experimental procedures, the U251 cells in the log phase of growth were utilized. The log phase represents the period of active cell proliferation, ensuring consistent and reliable results across subsequent tests. By employing these standardized cell culture techniques and maintaining the cells in optimal conditions, we ensured the availability of viable U-251 glioblastoma cells for our experiments, allowing for the reliable investigation of temozolomide sensitivity and resistance mechanisms.

**III.4. Transfection of siRNA**

In order to investigate the functional role of SP100 gene in U-251 glioblastoma cancer cells, we employed the Gene Pulser electroporation device for transfection. The specific siRNA targeting SP100 (Santa Cruz, USA) was utilized for gene knockdown.

The transfection process was conducted following the manufacturer's instructions provided by Bio-Rad. Briefly, U-251 cells were harvested and resuspended in an appropriate electroporation buffer. The siRNA and cell suspension were mixed together, and the mixture was transferred to an electroporation cuvette. The cuvette containing the cell-siRNA mixture was then placed in the Gene Pulser electroporation device.

To optimize transfection efficiency, we applied specific electroporation parameters. The settings used were TC = 12.5 ms and voltage = 160 V with a square wave. These parameters were determined based on preliminary experiments and established protocols for efficient gene delivery into U-251 cells. Following electroporation, the transfected cells were immediately transferred to various cell culture plates or dishes, depending on the specific assays or experiments being conducted. The choice of culture

plates was based on the requirements of the downstream analyses and assays. Subsequent experimental procedures were performed according to the specific assays and endpoints of interest. The transfected cells were incubated under appropriate culture conditions, including a humidified incubator at 37 degrees Celsius with 5% carbon dioxide. The transfection of siRNA allowed for the targeted knockdown of the SP100 gene in U-251 glioblastoma cells, providing a valuable tool to investigate the functional consequences of SP100 depletion in the context of glioblastoma.

**III.5. Quantitative Real-time Polymerase Chain Reaction and Gene Expression**

To assess the gene expression levels of SP100 and TRIM29 in U-251 glioblastoma cells, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. The primer sequences used for the amplification were obtained from Bioneer (South Korea) and are listed in Table 3.

U-251 glioblastoma cells were seeded in 6-well plates at a density of 4×10^5 cells per well, using plates from SPL (South Korea). The cells were allowed to adhere and grow until reaching the desired confluency. Total RNA was extracted from the cells using the Trizol reagent, specifically the RiboEx Kit from GeneAll (Korea). The Trizol reagent is a widely used method for efficient RNA isolation from various biological samples. Following the extraction, cDNA synthesis was performed according to the protocol provided within the kit, utilizing the cDNA synthesis components from Biofact (Korea).

To quantify the expression levels of SP100 and TRIM29 genes, qRT-PCR was carried out using the SYBR Premix Ex Taq from Biofact. The StepOne Plus real-time PCR equipment from Applied Biosystems (Thermo Fisher Scientific, USA) was employed for the amplification and detection of the PCR products. This system allows for real-time monitoring of the amplification process, enabling accurate quantification of gene expression levels. GAPDH, a commonly used housekeeping gene, was utilized as an internal control for normalization purposes. It helps account for variations in RNA input and cDNA synthesis efficiency, ensuring accurate comparisons between different samples.

The mRNA expression levels of SP100 and TRIM29 genes were determined using the 2-ΔΔct method. This method involves calculating the fold change in gene expression relative to a control or reference sample, based on the threshold cycle (Ct) values obtained during qRT-PCR. By employing qRT-PCR and the 2-ΔΔct technique, we were able to quantitatively analyze the expression levels of SP100 and TRIM29 genes in U-251 glioblastoma cells. This analysis provides valuable insights into the potential involvement of these genes in glioblastoma and their potential roles as biomarkers or therapeutic targets.

**Table 3:** The primer pairs used in the study.

| Sequence of primers | Primer | Gene name |
|---|---|---|
| *5′-AAGGATGCCGTGGATAAATTGC-3′*<br>*5′- ACACGATGAACTCACTGAAGTC-3′* | *Forward*<br>*Reverse* | *SP100* |
| *5′-CTGTTCGCGGGCAATGAGT-3′*<br>*5′-TGCCTTCCATAGAGTCCATGC-3′* | *Forward*<br>*Reverse* | *TRIM 29* |
| *5′-CTGGGCTACACTGAGCACC -3´*<br>*5′- AAGTGGTCGTTGAGGGCAATG-3´* | *Forward*<br>*Reverse* | *GAPDH* |
| *5′-CCGUGGAUAAAUUGCUCAAdTdT -3´*<br>*5′ -UUGAGCAAUUUAUCCACGGdTdT-3´* | *Sense*<br>*Anti-Sense* | *SP 100 siRNA* |

**III.6. MTT Assay**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to assess the cytotoxic effects of Temozolomide on glioblastoma cancer cells. This colorimetric assay is commonly used to measure cell viability and proliferation. U-251 glioblastoma cells were seeded in 96-well plates at a density of 1.2 × 10^4 cells per well and allowed to adhere and grow overnight. The cells were then treated with various concentrations of the drug candidate, Temozolomide, ranging from 0.01 to 100 μM. This concentration range was chosen to evaluate the dose-dependent response of the cells to the drug.

Following a 48-hour incubation period, the MTT assay was performed. The MTT solution, prepared at a concentration of 5 mg/ml, was added to each well (20 μl per well). The cells were further incubated for 4 hours to allow the conversion of the yellow tetrazolium salt, MTT, into purple formazan crystals by metabolically active cells. After the incubation period, the formazan crystals were dissolved by adding 100 μl of dimethyl sulfoxide (DMSO) to each well. This step solubilized the formazan crystals, resulting in a colored solution proportional to the number of viable cells. The absorbance of the solution was measured using a microplate reader (Tecan, Switzerland) at a wavelength of 570 nm, with a reference wavelength of 620 nm to account for background noise and non-specific absorbance.

All experiments were performed in triplicate to ensure reliable and reproducible results. The average absorbance values were used to quantify cell viability and determine the

cytotoxic impacts of Temozolomide on the U-251 glioblastoma cells. By conducting the MTT assay, we were able to assess the effect of Temozolomide treatment on the viability and proliferation of glioblastoma cancer cells. The results obtained from this assay contribute to our understanding of the drug's efficacy and provide valuable information for further investigations and potential clinical applications.

### III.7. Development of Temozolomide-Resistant Cells

To investigate the development of resistance to temozolomide in glioblastoma cells, we employed a gradual exposure approach. U251 cells were subjected to increasing concentrations of temozolomide over several months to induce resistance. The final concentration of temozolomide used for this purpose was 100 μM, which represents a clinically relevant concentration. Initially, U251 cells were cultured in regular media without the presence of temozolomide to establish a control group. In parallel, another set of U251 cells was exposed to incrementally increasing concentrations of temozolomide. The concentration was gradually escalated over time, allowing the cells to adapt and develop resistance to the drug. During the selection process, the temozolomide-resistant cells were continuously cultured in media containing the final concentration of 100 μM temozolomide. This ensured the maintenance and stability of the acquired resistance phenotype. The control cells, on the other hand, were cultured without temozolomide to serve as a comparison group.

The temozolomide-resistant cells, along with the control cells, were subsequently utilized for further experiments and analyses. By comparing the resistant cells to the control cells, we aimed to identify and characterize the molecular and phenotypic changes associated with temozolomide resistance in glioblastoma. The development of temozolomide-resistant cells provides an invaluable model system for studying the mechanisms underlying drug resistance in glioblastoma. These cells allow for the exploration of altered signaling pathways, genetic changes, and potential therapeutic strategies to overcome resistance and enhance treatment efficacy.

### III.8. Statistical Analysis

In this investigation, rigorous statistical analysis was performed to ensure the reliability and significance of the obtained results. Multiple independent assessments were conducted for each dataset to minimize experimental variability. Descriptive statistics, such as the mean and standard deviation, were calculated based on the measurement data to summarize the central tendency and variability of the samples.

To evaluate group differences and similarities, statistical tests such as t-tests or analysis of variance (ANOVA) were employed, depending on the nature of the comparisons. These tests allowed us to determine the statistical significance of observed differences between experimental groups. The choice of specific statistical tests was based on the experimental design and research questions. The statistical analysis was performed using GraphPad Prism version 8 software. This software is widely used for statistical

analysis and data visualization in biomedical research. A p-value of 0.05 was set as the threshold for statistical significance, indicating a 5% probability of obtaining the observed results by chance alone. Results with p-values below this threshold were considered statistically significant.

In addition to conventional statistical analysis, bioinformatics analysis was also conducted using R version 4.2.3, a powerful programming language and software environment for statistical computing and graphics. Various packages, including LIMMA, GEOquery, Biobase, and ggplot2, were utilized for bioinformatics analysis. These packages provide a range of functions and tools for data manipulation, differential gene expression analysis, visualization, and interpretation. The combination of statistical analysis using GraphPad Prism and bioinformatics analysis using R allowed us to comprehensively analyze and interpret the data obtained from different experiments and datasets. This integrated approach enabled us to draw meaningful conclusions, identify significant findings, and gain deeper insights into the investigated phenomena.

## IV. Results

### IV.1. DEG Analysis

We performed differential gene expression analysis to identify genes that were differentially expressed between temozolomide-sensitive and resistant U251 cells. The normalized expression values for the samples are shown in Fig. 7A. We identified a total of 591 differentially expressed genes (DEGs) using the mentioned criteria (Fig 7. 2B). Of the 591 DEGs, 263 genes were upregulated and 328 genes were downregulated in TMZ-resistant cells compared to temozolomide-sensitive cells. These genes may play important roles in the development of resistance to temozolomide in glioblastoma cells and may represent potential therapeutic targets for overcoming drug resistance.

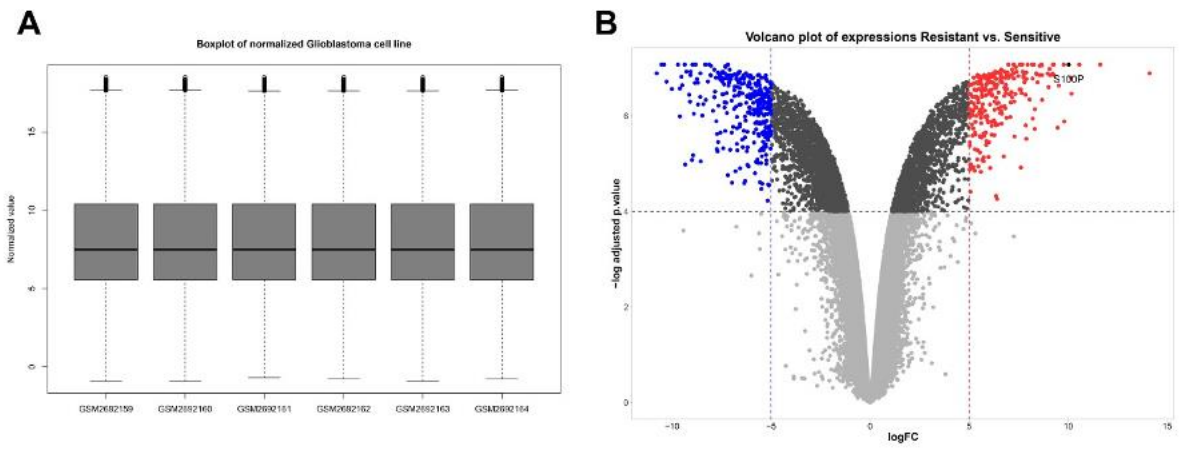


**Fig. 7** (A) Boxplot showing the normalized expression values for the samples. The box represents the interquartile range (IQR), the line within the box represents the median, and the whiskers extend to 1.5 times the IQR. (B) Volcano plot showing the DEGs between temozolomide-sensitive and resistant U251 cells. The x-axis

represents the logFC in expression, and the y-axis represents the negative log10 of the adjusted p-value. The red dots represent upregulated DEGs, the blue dots represent downregulated DEGs, and the gray dots represent non-significant genes. The dotted lines represent the cutoffs for statistical significance (|logFC| >= 5 and adjusted p-value < 0.0001).

### IV.2. S100P and their co-expressed DEGs

To explore the co-expression relationships among the DEGs identified in our study, we constructed a co-expression network using the GeneMANIA plugin in Cytoscape (Fig.8 2A). The purple lines in the network indicate highly co-expressed DEGs in a common network. We identified S100P as the gene with the highest degree in the network, with 45 interactions with other DEGs. To further explore the potential functional relationships of S100P with its co-expressed DEGs, we extracted the direct neighbors of S100P in the network and analyzed them as a separate network. The results showed that TRIM29 was the second-highest ranked gene in this network, with 23 interactions and a direct interaction with S100P (Fig. 8B).

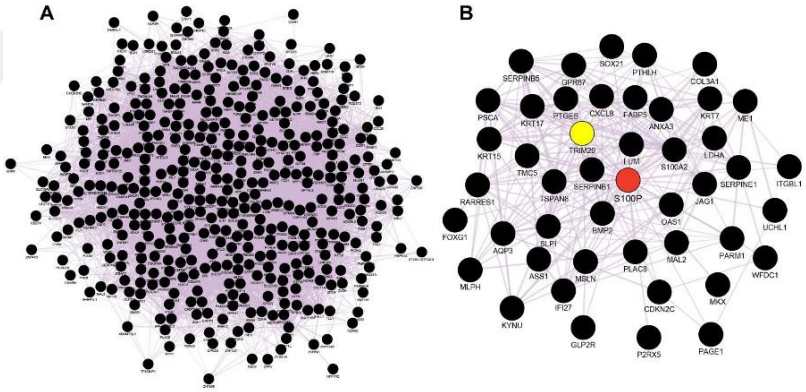


**Fig. 8** (A) Co-expression network of DEGs identified in our study. The purple lines indicate highly co-expressed DEGs in a common network. S100P is shown as a red oval and TRIM29 is shown as a yellow oval. (B) Network of the direct neighbors of S100P in the co-expression network shown in panel A. S100P is shown as a red oval and TRIM29 is shown as a yellow oval. The purple lines indicate the interactions between the direct neighbors of S100P.

### IV.3. IC50 analysis of parental and resistant U251 cells

To investigate the effect of temozolomide resistance on U251 cells, we performed IC50 analysis on both parental and resistant cells. We found that the IC50 value of temozolomide in the parental U251 cells was 11.93 μM, while in the resistant U251

cells, it increased to 91.66 μM, indicating that the resistant cells were significantly less sensitive to temozolomide treatment. To further explore the role of S100P in temozolomide resistance, we transfected resistant U251 cells withS100P siRNA and performed IC50 analysis. We found that the IC50 value decreased to 14.3 μM, indicating that downregulation of S100P sensitized the resistant cells to temozolomide treatment (Fig. 9).

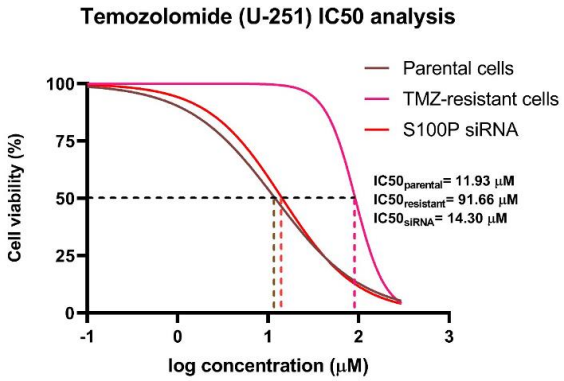


**Fig. 9** Non-linear regression analysis of temozolomide dose-response curve in parental and resistantU251 cells with or without S100P siRNA transfection.

### IV.4. Altered expression of S100P and TRIM29

To investigate the effect of temozolomide resistance on the expression of S100P and TRIM29, we performed qRT-PCR analysis on parental, resistant, and resistant cells transfected with S100P siRNA. Our results showed a significant increase in the expression levels of S100P and TRIM29 in the resistant cells compared to parental cells ($p < 0.05$). Interestingly, upon transfection of S100P siRNA, the expression levels of both S100P and TRIM29 significantly decreased compared to the resistant and parental cells ($p < 0.05$). These findings suggest that S100P may play a crucial role in the upregulation of TRIM29 in temozolomide-resistant U251 cells (Fig. 10).

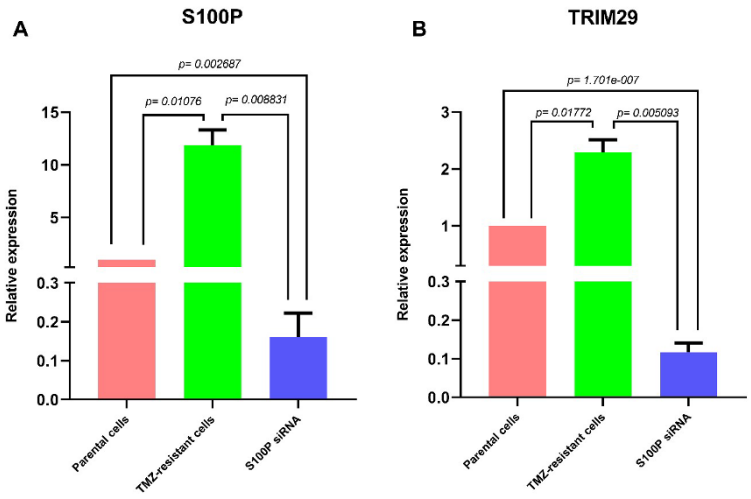


**Fig. 10** Expression levels of S100P and TRIM29 in parental, resistant, and S100P siRNA transfected cells. (A) qRT-PCR analysis showed a significant increase in the expression level of S100P in resistant cells compared to parental cells ($p < 0.05$). Transfection of S100P siRNA resulted in a significant decrease in the expression level of S100P compared to resistant and parental cells ($p < 0.05$). (B) qRTPCR analysis showed a significant increase in the expression level of TRIM29 in resistant cells compared to parental cells ($p < 0.05$). Transfection of S100P siRNA resulted in a significant decrease in the expression level of TRIM29 compared to resistant and parental cells ($p < 0.05$).

## V. Discussion

Glioblastoma (GBM) is the most common and lethal primary brain tumor in adults, with a median survival period of less than two years, despite advances in surgery, radiation treatment, and chemotherapy [145, 146]. Temozolomide (TMZ) is a chemotherapy medicine commonly used to treat GBM. Unfortunately, not all patients benefit from it, and one of the biggest problems in treating GBM is TMZ resistance [147,148].

There are several mechanisms underlying TMZ resistance, and one of them is the overexpression of the calcium-binding protein S100P [149]. S100P is a nuclear protein that controls the expression of genes and DNA repair [150]. Overexpression of S100P in GBM cells may cause resistance to TMZ by promoting DNA repair and reducing the production of O6-methylguanine (O6MeG), a DNA adduct responsible for temozolomide's cytotoxicity [146, 152].

Various strategies have been proposed to overcome TMZ resistance in GBM, including targeting the expression or function of the S100P protein. In a recent study, researchers investigated the impact of S100P on TMZ resistance in glioblastoma, as well as the potential of this protein as a therapeutic target [153]. In the study, the researchers used

computational bioinformatics analysis of gene expression and siRNA to target S100P protein expression levels in order to identify the mechanisms that may prevent TMZ resistance in GBM. They also investigated the possible relationship between S100P and TRIM29 in the development of TMZ resistance in glioblastoma cells. The results showed that TMZ was more effective in preventing tumor development in vitro when S100P was suppressed, indicating that targeting S100P may be a useful strategy for overcoming TMZ resistance in glioblastoma [154]. However, it is important to note that S100P is not the only component that plays a role in determining TMZ resistance, and combination treatments that focus on different pathways may be required to overcome resistance [155].

Additionally, the research was carried out with the use of cell lines and bioinformatics, and it is not yet known whether the findings can be applied to real-life situations involving actual individuals. Targeting S100P in glioblastoma patients will need more clinical testing to evaluate safety and effectiveness. Despite these limitations, the study's findings shed new light on the molecular mechanisms of glioblastoma and may contribute to the development of more effective treatments. Further research is needed to fully investigate the therapeutic potential of S100P inhibitors and to determine the safety and efficacy of this approach. In conclusion, targeting S100P may be a promising strategy to overcome TMZ resistance in glioblastoma, but more research is needed to fully explore this potential therapeutic avenue.

## VI. Conclusion

In conclusion, the findings of this study demonstrate the potential for targeting the calcium-binding protein S100P as a promising strategy to overcome Temozolomide resistance in glioblastoma. The results reveal that silencing S100P can sensitize glioblastoma cells to Temozolomide treatment, ultimately enhancing its efficacy. The implications of these findings are significant, as they not only provide new insights into the underlying mechanisms of Temozolomide resistance, but also offer a potential therapeutic avenue for the treatment of glioblastoma. Glioblastoma is a highly aggressive form of brain cancer that has proven difficult to treat. Despite advances in treatment options, including the use of Temozolomide, the five-year survival rate for patients with this disease remains low. One of the main obstacles to effective treatment is the development of resistance to chemotherapy drugs like Temozolomide. Therefore, identifying new targets for therapy is critical to improving outcomes for patients with glioblastoma.

The results of this study suggest that S100P may play a critical role in Temozolomide resistance in glioblastoma. S100P is a calcium-binding protein that has been implicated in various cellular processes, including proliferation, migration, and invasion. Previous studies have shown that S100P is overexpressed in a variety of cancers, including glioblastoma, and that its expression is associated with poor prognosis. In this study,

we used RNA interference to silence S100P expression in glioblastoma cells and examined the effects on Temozolomide sensitivity. The results showed that S100P silencing significantly increased the sensitivity of glioblastoma cells to Temozolomide treatment, suggesting that S100P may be a key mediator of Temozolomide resistance in these cells.

Importantly, the findings of this study have potential implications for the development of new therapeutic approaches for glioblastoma. By targeting S100P, it may be possible to sensitize glioblastoma cells to Temozolomide and enhance its efficacy. This approach could potentially overcome Temozolomide resistance and improve treatment outcomes for patients with glioblastoma. However, further research is needed to fully investigate the molecular mechanisms of S100P-mediated Temozolomide resistance and to explore the therapeutic potential of S100P inhibitors in glioblastoma treatment. It will be important to determine whether S100P is a general mediator of chemotherapy resistance in glioblastoma or whether it is specific to Temozolomide. Additionally, the development of S100P inhibitors will require careful evaluation to ensure their safety and efficacy.

In summary, this study provides valuable insights into the mechanisms of Temozolomide resistance in glioblastoma and offers a potential therapeutic avenue for the treatment of this devastating disease. By targeting S100P, it may be possible to enhance the efficacy of Temozolomide and overcome resistance, ultimately improving outcomes for patients with glioblastoma. While further research is needed to fully explore the therapeutic potential of S100P inhibitors, this study represents an important step forward in the ongoing effort to improve treatment options for patients with this challenging disease.

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