RESEARCH ARTICLE

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In Silico and *In Vitro* Study of mRNA Biomarkers for Glioblastoma Multiforme Resistance to Temozolomide (TMZ): The Association with Stemness

Veronica Hesti Candraningrum[1], Septelia Inawati Wanandi[2,3]*, Rafika Indah Paramita[4,5]

Abstract

Background: Glioblastoma multiforme (GBM) is an aggressive brain tumor that primarily affects adults. The Stupp Protocol, which includes surgical resection, chemoradiation, and monotherapy with temozolomide (TMZ), is the standard treatment regimen for GBM. However, repeated use of TMZ leads to resistance in GBM cells, resulting in a poor prognosis for patients. This resistance is driven by several intrinsic factors. This study aims to identify potential biomarkers of resistance associated with stemness. **Methods:** We utilized datasets from GEO, performed Venn diagram intersection analysis, conducted GO enrichment analysis using DAVID and ENRICHR, carried out pathway enrichment analysis with KEGG and REACTOME, and executed survival analysis with GEPIA. Additionally, we compared mRNA expression using the Human Protein Atlas and validated our findings with qRT-PCR. **Results:** We identified that PAQR6 and ITPKB mRNA expression was consistently higher in TMZ-resistant T98G cells, but TGFBI mRNA expression was found to be significantly higher in TMZ-resistant T98G cells compared to U87MG cells. In addition, a significantly higher CD133 mRNA expression as a stemness marker was found in T98G cells compared to U87MG cells. It is hoped that the acquired disease-related resistance biomarker candidates will be able to be used at the clinical level in terms of non-invasive early detection in GBM patients. However, additional research is required to validate the findings of this preliminary biomarker discovery study.

Keywords: DEG- resistance- stemness- glioblastoma- enrichment

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Introduction

Glioblastoma multiforme (GBM) is classified as an aggressive tumor of the brain and often attacks adults. This disease has a low incidence rate globally, namely around 5/100,000, when compared with other malignant tumors [1, 2]. Statistical data obtained from WHO states that GBM is ranked 15th in Indonesia and there were 5964 new cases recorded in 2019 accompanied by a death rate of 5298 people. Ostrom et al. [3] stated that glioblastoma accounts for as much as 15% of total central nervous system tumours and approximately 45% of primary malignant brain tumors. Various research efforts have been made to deepen and treat this tumor, but the prognosis for GBM is still poor with the average survival of sufferers being 12-15 months [3].

The treatment used to treat GBM is known as the Stupp Protocol [4, 5] which begins with surgical resection, chemoradiation and mono-agent chemotherapy therapy. Surgical resection is performed to remove GBM tumour tissue. The treatment steps are continued with a combination of radiation and chemical therapy (chemoradiation) for 6 weeks which aims to eliminate the remaining tumour cells. The chemical drug used is Temozolomide (TMZ) at a dose of 75 mg/m2 of body surface area. After chemoradiation treatment is completed, therapy is paused for 4 weeks and continued with consumption of TMZ as a single drug at a higher dose than combination therapy, as much as 150-200 mg/m2 of body surface area for 6 cycles. This technique is a standard treatment regimen for patients diagnosed with GBM [6].

Temozolomide (TMZ) is the most common chemotherapy drug used to treat patients with GBM [1, 6, 7]. This oral drug acts as an alkylating agent whose

*[1]Master's Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta Indonesia. [2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. [3]Molecular Biology and Proteomics Core Facilities (MBPCF), Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. [4]Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. [5]Bioinformatics Core Facilities (BCF), Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. *For Correspondence: septelia.inawati@ui.ac.id*

antitumor activity was first recorded in 1987 and its efficacy was validated by the FDA in March 2005 on the Stupp protocol. The combination of TMZ with radiation therapy has a better impact compared to radiotherapy treatment alone, such as extending patient survival two years longer than average and accompanied by an improvement in quality of life. The main mechanism of TMZ is to alkylate tumor genomic DNA, resulting in cytotoxic activity that can induce GBM tumor death. The alkylation activity is in the form of transporting methyl groups attached to guanine at positions O6 and N7, and adenine at position N3 during DNA replication to form cytotoxic O6-methylguanine (O6-MG), N7-methylguanine (N7-MG), and N3-methyladenine (N3MA). These cytotoxic groups consist of lethal base pair mismatches that produce single- and double-strand DNA breaks [1, 7, 8].

The effect of using TMZ in treating GBM was noted to be successful due to its cytotoxic activity, however resistance effects were found in several cases. Perazzoli et al. [9] stated that the failure in TMZ therapy was the expression of the O6-methylguanine-DNA methyltransferase (MGMT) gene as a response to repairing damaged cells. MGMT is a DNA repair enzyme with a mechanism for repairing the main cytotoxic lesions caused by TMZ. The enzyme works by removing the methyl group in O6-methylguanine, thereby canceling the cytotoxic process that should occur as an effect of TMZ. Various studies have shown that TMZ resistance is a result of overexpression of MGMT as well as other genes such as ATRX, IDH1, H3F3A, and TERT which are involved in DNA repair [10, 11]. Other factors such as mutations in the MMR (mismatched repair) pathway9, autophagy, and cell reprogramming by GBM stem cells (GSC) also contribute to the occurrence of TMZ resistance [6]. Clarification of TMZ resistance mechanisms is essential to improve drug efficacy and can provide an important basis for precision medicine according to different resistance mechanisms.

GSCs are a collection of GBM stem cells that actively carry out tumorigenesis, differentiation, and are able to determine heterogeneous cell phenotypes depending on the micro environment through cell reprogramming mechanisms [6]. The GSC population contributes to chemotherapy resistance and cancer recurrence [6, 8, 9, 12, 13]. This is because TMZ administration induces the growth of cell populations such as cancer stem cells with enriched CD133+ markers, which is thought to be mediated by upregulation during the DNA repair process [8, 14]. In addition, GSCs have the ability to self-renew and differentiate into heterogeneous cancer cell lineages in response to chemotherapeutic agents [6, 13, 15].

The expression of specific resistance genes related to stemness markers and their protein interactions remains largely unknown. To address this, our research will analyze specific resistance genes using gene expression datasets from resistant primary cells and the T98G resistant cell line. The intersection of these datasets will be further analyzed with a resistant stem cell gene expression dataset. This approach aims to identify specific resistance-related genes associated with stemness markers that can intrinsically alter the GBM response from sensitive to resistant

Materials and Methods

Dataset Selection and Preparation

The analysis series began with a search for specific genes resistant to TMZ in the GEO database with the keywords “Human”, “Glioblastoma”, “Resistance”, “Sensitive”, “Glioma stem cell”, and “glioma full stem like phenotype”. The dataset group was divided into resistance group and stemness group. The results of the GEO2R analysis were then downloaded and analysed with Microsoft Excel to obtain a list of genes that increased by applying a log2FC≥1 cut off filter for increased gene expression analysis and log2FC≤-1 for decreased gene expression analysis (Padj <0.05).

Overlapping DEGs Analysis

A number of differentially expressed genes (DEGs) were separated and further analyzed to obtain the DEG fragments found in each group. This stage uses the web-based tool InteractiVenn (http://www.interactivenn.net) [16].

Biomarker Validation through RT-qPCR

The cells used in the wet lab test were 1) GBM T98G cells (ATCC CRL-1690) obtained from Prof. Alexander Brehm from the Institut für Molekularbiologie und Tumorforschung (IMT) Marburg, Germany, and 2) GBM U87MG cells (ATCC HTB-14) obtained from Dr. Christine Banjarnahor. The medium used was DMEM medium [Gibco, Thermo Fisher Scientific] which had been supplemented with both Penicillin 1% and Streptomycin 1% [Gibco, Thermo Fisher Scientific]. The amplification process requires primers from three DEGs designed with Primer-BLAST. The amplification process was carried out following the kit protocol used [Vazyme, 2023], with a different annealing temperature for each primer pair (Appendix 1). Gene expression was normalized to 18s rRNA as HKG in this study. The CT values obtained from RT-qPCR were then analyzed for relative expression using the Livak method along with by graphs created with Microsoft Excel and statisctical analysis through JASP.

Enrichment Analysis

Functional analysis was performed using DAVID (https://david.ncifcrf.gov/) on the identified DEGs. Enrichment analysis for gene ontology and signaling pathways was conducted on sliced proteins and those outside the slices using KEGG (https://www.kegg.jp/kegg/pathway.html) and REACTOME (https://reactome.org/), both integrated within the ENRICHR platform (https://maayanlab.cloud/Enrichr/). Information on gene location, gene size, and protein characterization for each biomarker was retrieved from the GeneCards database (https://www.genecards.org/). The Human Protein Atlas (https://www.proteinatlas.org/) was utilized to compare biomarker RNA expression levels in GBM with other cancer types. Finally, survival analysis was carried out via the Gene Expression Profiling Interactive Analysis (GEPIA) platform (http://gepia.cancer-pku.cn/), where differential expression and

survival analysis of hub genes were performed between tumor and healthy groups. The Kaplan-Meier survival analysis, using a 50% expression cut-off, compared high and low expression groups.

Results

Dataset Selection and Preparation

Four datasets were successfully obtained from GEO and divided into two groups, namely the GBM resistance group and the GBM stemness group. The GSE62153 and GSE193957 datasets were chosen as the GBM resistance group because both datasets contain gene expression samples from resistant cells and sensitive cells. In the GBM stemness group, the GSE23806 and GSE124145 datasets were used. These two datasets were chosen because they have gene expression samples from GBM stem cells and general GBM sustaining cells. Specific information regarding the use of each dataset can be seen in Table 1.

Analysis with the GEO2R tool was carried out on each dataset to obtain a list of genes that increased or decreased. In GSE62153, it was found that 384 genes had increased expression and 124 genes had decreased expression. In GSE193957, it was found that 1501 genes had increased expression and 1410 genes had decreased expression. In GSE23806, it was found that 2672 genes had increased expression and 2792 genes had decreased expression. In GSE124145, it was found that 2037 genes had increased expression and 2261 genes had decreased expression (Figure 1).

Overlapping DEGs

Intersection analysis was carried out on each gene that increased and each gene that decreased using InteractiVenn. The results of the intersection of the two groups resulted in two genes in the intersection of both groups increasing and one gene in the intersection of the second group decreasing. The PAQR6 and ITPKB genes were genes found to be increased in the group, while TGFBI was a gene found in slices of both groups that had decreased expression (Figure 2). These three genes are then referred to as DEG markers of resistance related to stemness.

Relative Expression Analysis

Sequences of two genes (PAQR6 and ITPKB) that were found to be candidate biomarkers of increasing stemness-related resistance and one gene (TGFBI) as a candidate biomarker of decreasing stemness-related resistance were searched on the NCBI website in the form of FASTA or accession ID. This information is then used in NCBI's Primer-BLAST page to search for the best primer pairs according to commonly known parameters. The results of the primary sequence design and the results of the primary annealing temperature optimization of the three genes were successfully obtained and are attached in Supplementary 2.

The next step was to amplify the target genes (PAQR6, ITPKB, and TGFBI) using one NC (Negative Control), 18S rRNA as HKG, and the CD133 gene as a primer from a commonly known stemness marker gene. The results of the relative expression test using Livak and the independent T-test statistical test (Figure 3) showed that the four biomarker genes were found to be higher in resistant T98G cells compared to TMZ sensitive U87MG cells.

Gene Onthology Analysis

Candidate biomarkers obtained in bioinformatics tests are PAQR6, ITPKB and TGFBI. These three DEGs were used in enrichment analysis using DAVID to determine biological processes (BP), cellular components (CC), and molecular function (MF) ($p<0.05$).

The pathway enrichment analysis contained in DAVID is then accessed via the EGG and REACTOME databases according to data availability. The results of enrichment analysis on the stem-related resistance biomarkers can only be carried out with DAVID (Table 2).

PAQR6

The PAQR6 gene (progestin and adipoQ receptor family member 6) or known as mPRδ is located on Chr1q22 with a size of ~4.7 kb. The PAQR6 protein consists of 344 amino acids with a molecular mass of 37.9 kDa (Gene Cards, 2023). PAQR6 is a gene that is a member of the G-protein coupled receptor (GPCR) group and is a stimulator protein of the G protein together with other PAQRs, namely PAQR5 (mPRγ), PAQR7 (mPRα), PAQR8 (mPRβ), and PAQR9 (mPRε) [17, 18]. Based on gene annotation analysis with DAVID (Table 2), it is known that PAQR6 in cellular component (CC) analysis shows significant enrichment in the plasma membrane and integral components of the membrane. Meanwhile, molecular function (MF) analysis showed significant enrichment in steroid binding, protein, and receptor signaling activity. Pathway analysis found for PAQR6 is in the neuroactive ligand-receptor interaction pathway in the

Table 1. Dataset used in This Research

Grouping	GEO Accession	Platform	Samples from dataset
Resistance GBM	GSE62153	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	18 primary resistant cells VS 25 primer sensitive cells
	GSE193957	GPL21185 Agilent-072363 SurePrint G3 Human GE v3 8x60K Microarray 039494	3 U87 Resistant cells VS 3 U87 Sensitive cells
Stemness GBM	GSE23806	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	12 glioma full stem-like phenotype (GSF) cell lines VS 32 GBM conventional cell lines
	GSE124145	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	6 glioma stem cell lines VS 3 GBM conventional cell lines

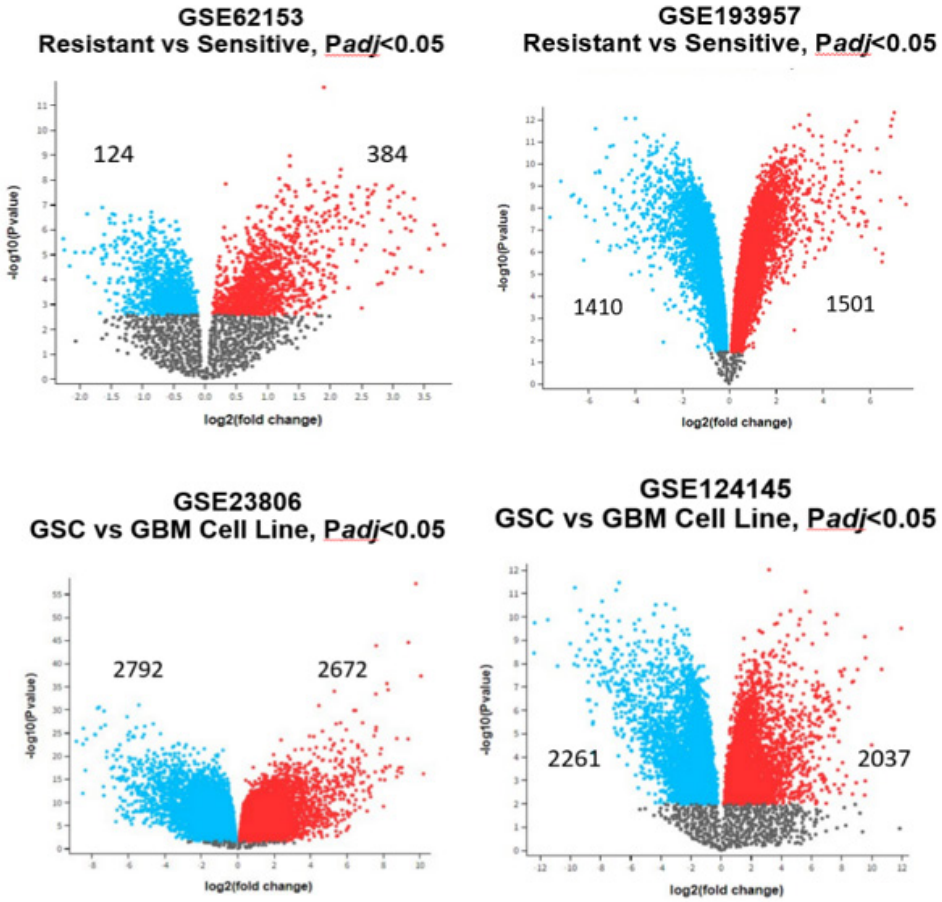

Figure 1. Volcano Plot on each Dataset; red=upregulated, blue=downregulated, black=insignificant

KEGG database (Figure 4). Silencing of PAQR6 is known to suppress the expression of MEK/ERK signaling which is involved in MAPK signaling [19]. Pathway analysis related to MAPK signaling was successfully downloaded from the KEGG database (Figure 5). KEGG results show that MAPK plays a role in the process of proliferation and differentiation of cancer cells.

The potential of PAQR6 as a biomarker in GBM was strengthened through Survival analysis in GEPIA and RNA expression in the Human Protein Atlas. The reduction in survival rates in patients with high levels of PAQR6 expression is predicted to decrease by less than 20% in a period of <30 months with a p value>0.05 (Supplementary Figure 6, left). The results of the RNA overview analysis in the Human Protein Atlas (TCGA database) using 153 glioma patient samples, obtained a median PAQR6 expression value of 27.8 FPKM as the highest value compared to PAQR6 expression from other types of cancer (Supplementary Figure 6, right). The results of analysis of PAQR6 RNA expression in TCGA strengthen the results of previous studies which stated that PAQR6 expression worsens the prognosis in prostate

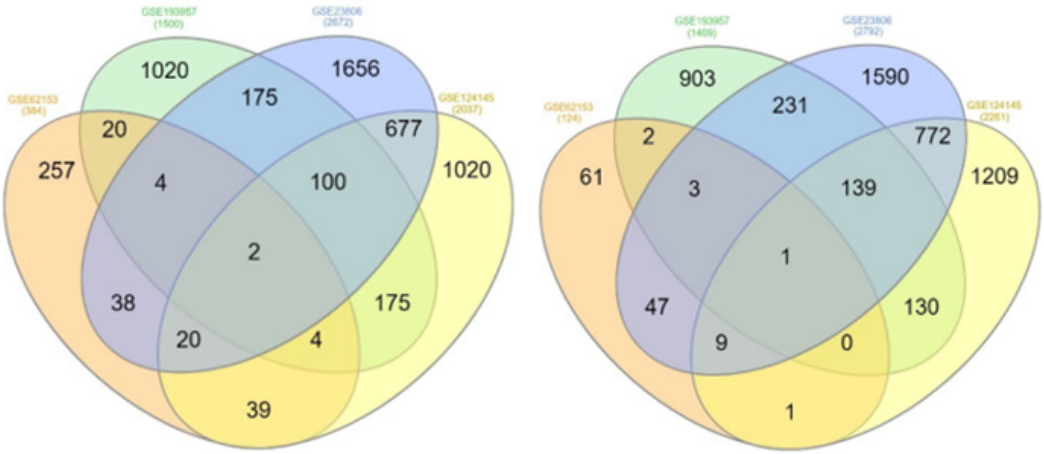

Figure 2. InteractiVenn Results Analysis; left=upregulated DEGs, right=downregulated DEGs

Table 2. Enrichment Analysis through DAVID ($p<0.05$)

ID	ITPKB	PAQR6	TGFBI
Gene Name	Inositol-triphosphate 3-kinase B	Progestin and adipoQ receptor family member 6	Transforming growth factor beta induced
Spesies	Homo sapiens	Homo sapiens	Homo sapiens
GOTERM_Biological Process	GO:0000165~MAPK cascade	-	GO:0001525~angiogenesis
	GO:0001932~regulation of protein phosphorylation		GO:0007155~cell adhesion
	GO:0002262~myeloid cell homeostasis		GO:0008283~cell proliferation
	GO:0007165~signal transduction		GO:0030198~extracellular matrix organization
	GO:0007166~cell surface receptor signaling pathway		GO:0050896~response to stimulus
	GO:0016310~phosphorylation		
	GO:0032957~inositol trisphosphate metabolic process		
	GO:0032958~inositol phosphate biosynthetic process		
GOTERM_Cellular Component	GO:0005634~nucleus	GO:0005886~plasma membrane	GO:0005576~extracellular region
	GO:0005737~cytoplasm	GO:0016021~integral component of membrane,	GO:0005604~basement membrane
	GO:0005783~endoplasmic reticulum		GO:0005615~extracellular space
	GO:0005829~cytosol		GO:0005886~plasma membrane
	GO:0005856~cytoskeleton		GO:0031012~extracellular matrix
	GO:0016020~membrane		
GOTERM_Molecular Function	GO:0000828~inositol hexakisphosphate kinase activity	GO:0005496~steroid binding	GO:0005178~integrin binding
	GO:0005515~protein binding	GO:0005515~protein binding	GO:0005201~extracellular matrix structural constituent
	GO:0005516~calmodulin binding	GO:0038023~signaling receptor activity	GO:0005515~protein binding
	GO:0005524~ATP binding		GO:0005518~collagen binding
	GO:0008440~inositol-1,4,5-trisphosphate 3-kinase activity		GO:0042802~identical protein binding
	GO:0016301~kinase activity		GO:0050839~cell adhesion molecule binding
	GO:0051765~inositol tetrakisphosphate kinase activity		GO:0050840~extracellular matrix binding
KEGG_Pathway	hsa00562: Inositol phosphate metabolism	hsa04080: Neuroactive ligand-receptor interaction	-
	hsa01100: Metabolic pathways		
	hsa04020: Calcium signaling pathway		
	hsa04070: Phosphatidylinositol signaling system		
REACTOME_Pathway	R-HSA-1430728~Metabolism,	-	R-HSA-392499~Metabolism of proteins
	R-HSA-1483249~Inositol phosphate metabolism,		R-HSA-977225~Amyloid fiber formation
	R-HSA-1855204~Synthesis of IP3 and IP4 in the cytosol,		

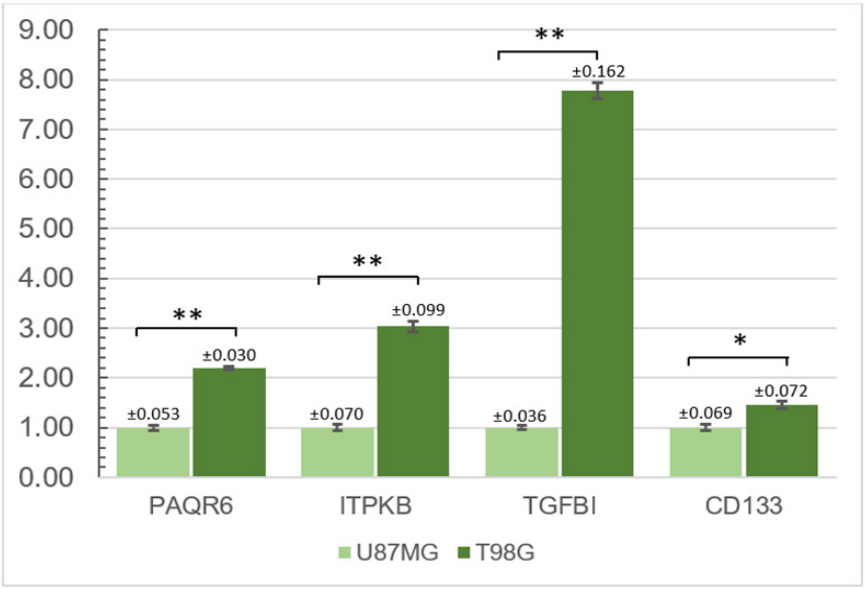

Figure 3. Expression Relative on Four Tested Genes (*p<0.05, **p<0.001)

cancer patients and strengthens the potential of PAQR6 as a marker of resistance associated with the onset of GBM.

ITPKB

The second stemness-related resistance marker gene obtained in bioinformatics analysis whose expression increased was ITPKB. The ITPKB (inositol-triphosphate 3-kinase B) gene or also known as IP3KB is located on Chr1q42.12 with a size of 107.5 kb. The ITPKB protein consists of 946 amino acids and measures 102.3 kDa (Gene Cards, 2023). This protein regulates the metabolism of inositol phosphates (IPs) through phosphorylation of the second messenger inositol 1,4,5-trisphosphate to

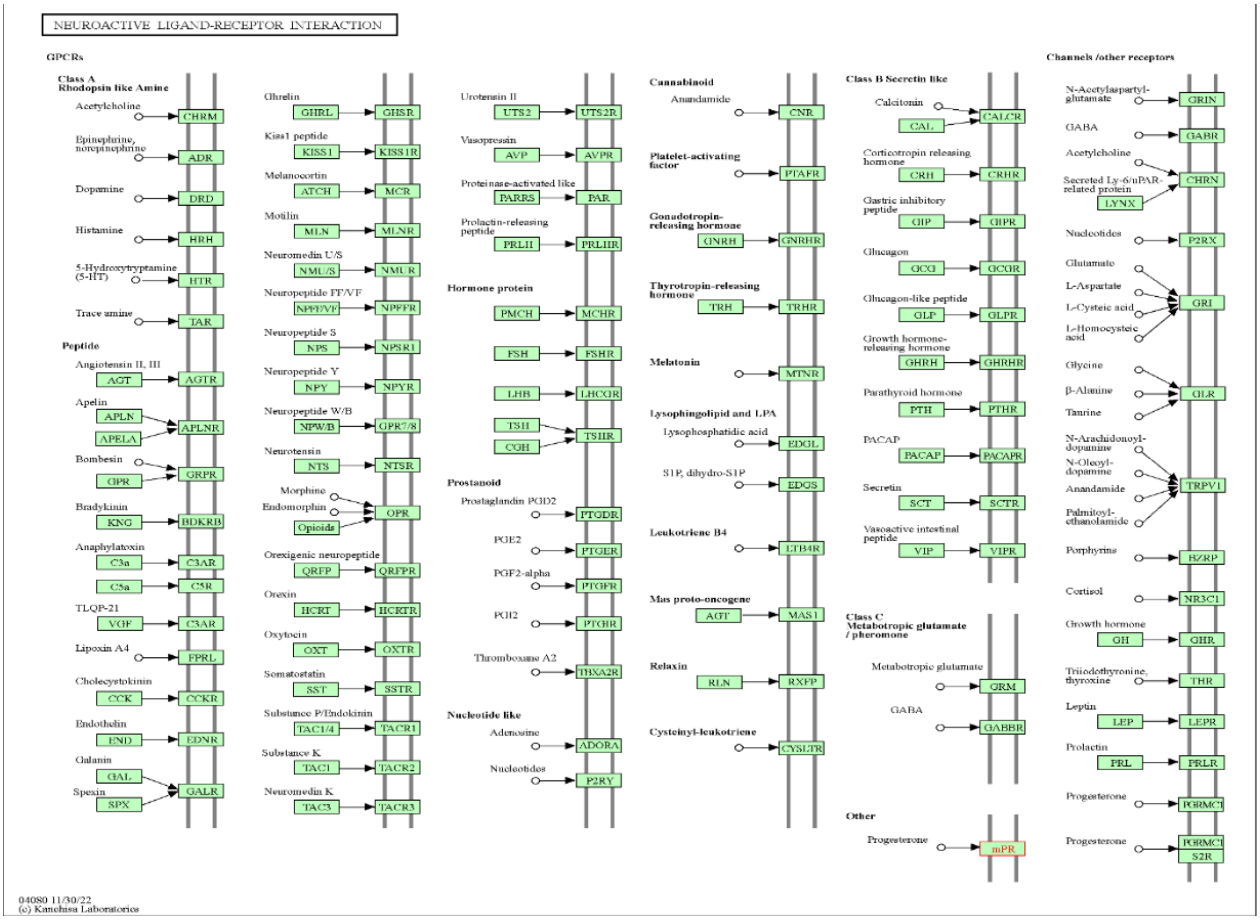

Figure 4. Analysis of PAQR6, which Belongs to the mPR Family of Proteins, is Involved in Neuroactive Ligand-Receptor Interactions

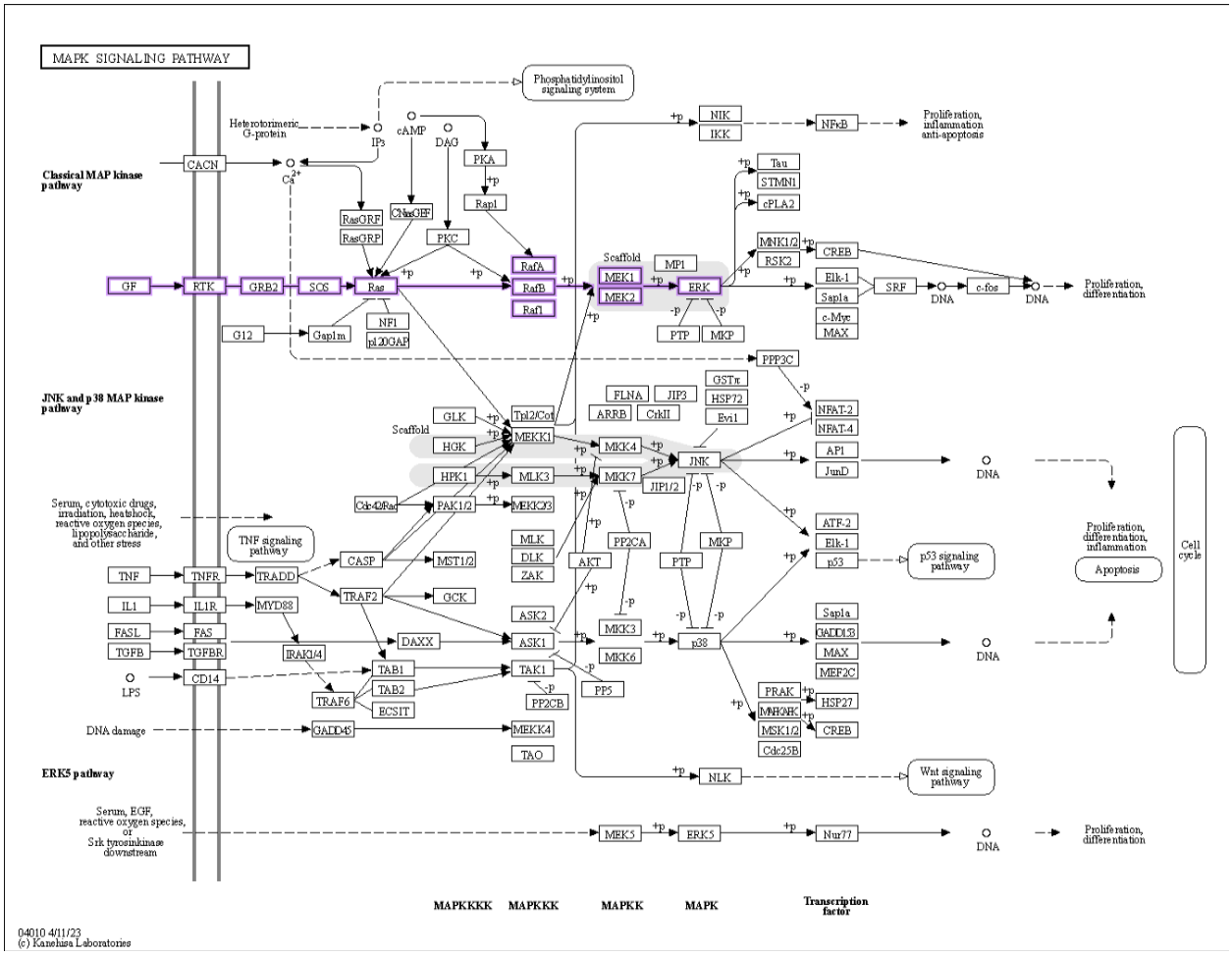

Figure 5. MAPK Signaling Associated with PAQR6 (KEGG, 2023) (highlighted in purple)

ins(1,3,4,5)P4. Inositol phosphate compounds regulate various intracellular processes, such as endo- and exocytosis, cell nuclear function, platelet aggregation, generation of reactive oxygen species (ROS), drug sensitivity, and viral replication [20].

The genes involved in IP metabolism were observed to be significant in biological processes (BP), involved in the MAPK signaling cascade process, regulation of protein phosphorylation, signal transduction processes, and even involved in the cellular response process to calcium ions. Meanwhile, in cellular component (CC) analysis, ITPKB showed significant enrichment in the nucleus, cytoplasm, endoplasmic reticulum and membrane. In molecular function (MF) analysis, ITPKB showed significant enrichment in inositol phosphate kinase activity, protein binding, calmodulin binding, and ATP binding. Pathway analysis showed significant enrichment in the inositol metabolism pathway (Supplementary Figure 7), calcium signaling, and the phosphatidylinositol signaling system. KEGG analysis was also added regarding IP compounds in the glioma signaling pathway (Supplementary Figure 8).

The potential of ITPKB as a biomarker in GBM was strengthened through survival analysis in GEPIA and RNA expression in the Human Protein Atlas. The reduction in survival rates in patients with high levels of ITPKB expression is predicted to decrease by up to 20% in a period of <20 months with a p value>0.05 (Supplementary Figure 9, left). The results of the RNA overview analysis in the Human Protein Atlas (TCGA database) using 153 glioma patient samples, obtained a median PAQR6 expression value of 24.5 FPKM as the second highest value after melanoma and compared to ITPKB RNA expression from other types of cancer (Supplementary Figure 9, right). The results of analysis of ITPKB RNA expression in TCGA strengthen the results of previous studies which stated that ITPKB expression worsens the prognosis in prostate cancer patients and strengthens the potential of PAQR6 as a marker of resistance related to the onset of GBM.

TGFBI

The TGFBI (transforming growth factor beta induced) gene is a candidate marker for disease-related resistance whose expression is reduced. This gene is located at Chr5q31.1 with a size of 34.8 kb. The protein encoded by this gene is a secreted protein with a molecular weight of ~68 kDa and consists of 683 amino acids (Gene Cards, 2023). TGFBI plays a role in cell attachment to each other (cell adhesion) and cell movement (migration). TGFBI, also known as βig-H3, is an extracellular matrix protein involved in various physiological and pathological processes, including cancer. The role of TGFBI in cancer is complex, with evidence suggesting both tumor suppressive and tumor promoting functions [21, 22].

GO analysis of BP shows that it is significantly involved in angiogenesis, chondrocyte differentiation, cell adhesion, and response processes to stimuli and localization. CC analysis showed significant enrichment in the extracellular area, basement membrane, extracellular space, trans-Golgi network, and extracellular exosomes. MF analysis showed significant enrichment in integrin binding, function in extracellular matrix structural constituents, protein binding function, and extracellular matrix binding. In pathway analysis with REACTOME, it was found that TGFBI was involved in the formation of amyloid fiber which is needed in protein metabolism. KEGG analysis of the signaling pathway by TGF-β is seen in Supplementary Figure 10.

The potential of TGFBI as a biomarker in GBM was strengthened through Survival analysis in GEPIA and RNA expression in the Human Protein Atlas. The reduction in survival rates in patients with high levels of TGFBI expression is predicted to decrease by up to 20% in a period of <20 months (Supplementary Figure 11, left). The results of the RNA overview analysis in the Human Protein Atlas (TCGA database) which used 153 glioma patient samples, obtained a median PAQR6 expression value of 21 FPKM and was not much different compared to TGFBI RNA expression from other types of cancer (Supplementary Figure 11, right).

Discussion

Resistance in GBM is a multifactorial event after TMZ administration which has been proven to reduce the survival rate in patients. Various responses in cells to carry out self-defense mechanisms, such as repairing damaged DNA structures, autophagy, growth of cancer stem cells, mutations in cell cycle regulatory genes, and other factors which in an integrated manner support the process of repeated emergence of post-GBM cells. giving TMZ. This research proves that there is a relationship between resistance and stemness in GBM, where there are resistance marker genes related to stemness. The results of this study also support previous theories which discuss the factors supporting the occurrence of resistance in GBM.

The analysis of the relative expression of four genes in RNA isolates from U87MG and T98G cells aligned with dry lab findings for PAQR6 and ITPKB, both identified as stem-related resistance marker genes with elevated expression in GBM. Specifically, PAQR6 showed a 2-fold increase, and ITPKB exhibited a 3-fold increase in expression in T98G cells compared to U87MG cells. The wet lab analysis yielded results that differed from those of the dry lab regarding TGFBI gene expression, revealing an approximately 8-fold increase in T98G cells compared to U87MG cells. Additionally, CD133, a well-known stemness marker gene, exhibited elevated expression levels, approximately 1.5 times higher in the resistant T98G cells than in the U87MG cells.

Resistance in stem-related GBM has been proven through this research by detecting the expression of candidate stem-related resistance marker genes obtained from bioinformatics analysis. This was confirmed by the expression of the CD133 gene which was significantly 1.46 times higher in TMZ-resistant T98G cells than in sensitive U87MG cells. The expression of the PAQR6 gene was significantly 2.20 times higher and the ITPKB gene was significantly 3 times higher in T98G cells than U87MG. In bioinformatics analysis, TGFBI was identified as a potential biomarker with reduced expression, but in RT-qPCR analysis using U87MG and T98G cells, it was found to have significantly higher expression in T98G cells. The inconsistency in results can be attributed to the variation in both the number and type of samples used in the bioinformatics analysis versus the RT-qPCR test. While DEG analysis in the stemness group was conducted using over 30 GBM cell lines, the RT-qPCR test was limited to only two, namely T98G and U87MG. Researchers propose that TGFBI expression levels observed in these two cell lines during RT-qPCR may not adequately reflect the broader GEO dataset analyzed.

The molecular mechanisms underlying the role of increased PAQR6 in stemness-associated GBM resistance are poorly understood. Several studies have suggested an association between PAQR6 expression and chemoresistance in prostate cancer, where increased expression of this gene is associated with poor prognosis and lower survival rates [17, 19]. PAQR6 belongs to the mPR family and the expression of this gene is closely related to progesterone in the body. Progesterone has been shown to have various effects on GBM development and resistance. Some studies show that progesterone has a dual role where one side can inhibit the growth of GBM cells, while other studies report that progesterone can promote cell migration and invasion [23]. Bello-Alvarez et al. [24] stated that progesterone and its derivative compound, allopregnanolone, were proven to promote migration and invasion of human GBM-derived cells. Bello-Alvarez et al. [25] also added that progesterone was shown to induce activation of the c-Src protein through the progesterone receptor (PR), and the interaction between PR and c-Src can regulate the activity of proteins involved in migration and invasion of human GBM cells.

The results of the enrichment analysis conducted on PAQR6 align with previous studies and corroborate the findings from the RT-qPCR test. PAQR6 is a membrane-associated mPR protein that has shown significant enrichment in membrane compartments. Masi et al. [26] added that activation of PAQR6 (mPR) signaling is caused by progestin so that it is able to support proliferation and migration in cancer cells via the MEK/ERK pathway and supports survival, invasion and proliferation in cancer cells via the PI3K/Akt pathway (Supplementary Figure 12). Progestin, which is a steroid hormone, will increase MAPK signaling [19] and MEK/ERK which supports the proliferation, migration and differentiation of cancer cells, as well as activating the PI3K/Akt pathway which supports survival, invasion and proliferation in cancer cells [26].

Neuroactive signaling found in KEGG as a significant pathway (Figure 4) has a role in GBM. Pal et al. [27] added that GBM patients combined with damage to the neuroactive ligand-receptor interaction pathway have a poor prognosis ($P<0.0001$). Monitoring these

signaling pathways can help predict tumor emergence and progression. The results of the survival analysis on ITPKB with GEPIA were not significant because the p value was> 0.05, so further examination was needed. The results of RNA detection in RT-qPCR are strengthened by an overview of PAQR6 RNA expression which was found to be high in Glioma according to the Human Protein Atlas and when compared with RNA expression from other types of cancer. PAQR6 was previously discovered by Li et al. [19] as a gene that can worsen the prognosis in prostate cancer. Thus, it does not rule out the possibility that PAQR6 has potential as a biomarker in GBM, especially regarding stem-related resistance.

The mechanism of direct ITPKB signaling in GBM resistance is not well understood. ITPKB is known to be a gene that codes for a protein that plays a role in IP metabolism (Supplementary Figure 7). Several studies have found that IP compounds play an important role in the development of GBM and resistance to treatment through various mechanisms, including the regulation of cell proliferation, survival, and migration, as well as the modulation of signal transduction and Ca2+ homeostasis in the central nervous system [28–32]. Moral-Morales et al. [18] added that the compound PDZ binding kinase (PBK), which is a kinase that is closely related to ITPKB, was identified as one of the therapy-resistant marker genes with significantly increased expression levels in GBM samples [33].

IP compounds are involved in various signaling pathways. An in-depth literature review successfully provided insights into the role of ITPKB in IP metabolism. Obeng et al. [34] added that activation of several phosphoinositide-specific phospholipase C (PLC) compounds will catalyze the production of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (ITP), where DAG and ITP regulate the activation of protein kinase C (PKC) and releases calcium ions into the cytosol. This process leads to the migration, defense, growth, and differentiation of cancer cells (Supplementary Figure 13) [34]. KEGG analysis related to signaling in Glioma (Supplementary Figure 8) involving IP compounds supports Obeng et al (2020), which clearly shows that there is a role for ITPKB which is involved in IP metabolism, where IP compounds are involved in the calcium signaling cascade that activates pathways. downstream such as Ras/Raf/MEK/ERK which supports the growth and proliferation of cancer cells, as well as activation of the PI3K/Akt pathway which leads to cancer cell survival.

The enrichment analysis of ITPKB reinforces the findings of previous studies and corroborates the results of the RT-qPCR test. ITPKB is a protein involved in the metabolism of IP compounds which is required in various pathways, specifically in calcium signaling and phosphatidylinositol signaling (Table 2, GO KEGG results on ITPKB). Calcium signaling that occurs due to PLC activation will activate PKC, where PKC will promote cell survival [34]. Phosphatidylinositol pathway obtained in KEGG results was found to be involved in the MAPK signaling cascade via CACN. Calcium ions that leave the intracellular space after the formation of second messengers InsP3 and DAG will activate Ras/Raf/MEK/ERK signaling, which supports proliferation and anti-apoptosis processes (Supplementary Figure 13). The results of RNA detection in RT-qPCR were strengthened by an overview of ITPKB RNA expression in glioma which was found to be the second highest after melanoma (Human Protein Atlas, 2023). The results of the survival analysis on ITPKB with GEPIA were not significant because the p value was> 0.05, so further testing was needed. However, researchers found that ITPKB has the potential to be a marker of resistance related to the onset of GBM based on the results of literature studies and bioinformatics tests.

The TGFBI signaling mechanism that supports chemoresistance is very complex and involves several pathways. TGFBI signaling is a signaling driver of epithelial-mesenchymal transition (EMT) which is a cellular program that confers stem cell-like properties and resistance to apoptosis. TGFBI signaling also promotes the development of cancer stem cells (CSCs), which is believed to be a major factor underlying therapy failure and chemotherapy resistance. Additionally, TGFBI signaling can induce the expression of secreted growth factors, such as platelet-derived growth factor (PDGF), which can activate pathways that promote chemoresistance. TGFBI signaling can also indirectly activate this pathway by inducing the expression of secreted growth factors. Additionally, TGFBI signaling has been shown to regulate cancer metabolic reprogramming, which is a key driver of chemoresistance [35–39].

The enrichment results strengthen previous studies that state the function and signaling mechanism of TGFBI which belongs to the TGF-β family. Biological processes involving TGFBI that are related to cancer resistance include angiogenesis, cell proliferation, and stimulus response. The results of the literature study obtained (Supplementary Figure 14) with KEGG TGF-beta analysis (Supplementary Figure 10) show that there is a relationship between PI3/Akt, ERK and Smad signaling with TGF-β [38, 40]. The KEGG pathway obtained strengthens the GO results on BP which state that the Smad signaling pathway plays a role in the angiogenesis process. Overactivation of TGF-β will activate the anti-apoptotic pathway via Smad, where KEGG analysis also shows signaling from Smad to MAPK. The MAPK pathway is known to play a role in proliferation, differentiation, and regulation of apoptosis. The pathway analysis found in TGFBI was also observed in PAQR6 and ITPKB. Survival analysis with GEPIA on TGFBI gave significant results ($p<0.05$), so it can be predicted that high TGFBI expression in patients with GBM will reduce the survival rate by up to 20% in less than 20 months (SupplementaryFigure 11, left). However, RNA expression data in the Human Protein Atlas depicts low RNA expression and is relatively similar to TGFBI expression in other types of cancer. This does not rule out the possibility of TGFBI as a marker of resistance related to the onset of GBM based on the results of literature studies and bioinformatics analysis.

CD133, or also known as prominin-1 (PROM-1) is a membrane-bound glycoprotein found on the surface of GSCs. Expression of the CD133 gene has been associated with poor prognosis, therapy resistance, and tumor

recurrence in glioblastoma multiforme (GBM). It has been found that chemotherapy drugs such as temozolomide, carboplatin, paclitaxel (taxol), and etoposide are not effective against CD133+ cells [41, 42]. Jang et al. [43] stated that the CD133 signaling mechanism in GBM is in the PI3K/Akt pathway, autophagy pathway, lipid metabolism, and ROS signaling mechanism pathway. The PI3/Akt pathway supports survival, cell proliferation and tumor cell growth. Activation of lipid metabolism will affect autophagy which plays a role in cell homeostasis, as well as signaling mechanisms by ROS which support chemotherapy resistance [43]. CD133 expression in this study was found to be significantly 1.5 times higher in resistant T98G cells, confirming previous research which found that CD133+ cells were resistant to TMZ. In addition, the results of this study strengthen previous research which found a relationship between CD133 expression and TGFBI. Recent studies found that CD133 regulates TGFβ signaling during cell repair processes in neurons and liver cells through Smad signaling. CD133 can interact with members of the signaling cascade and activate or contribute to the inhibition of TGFβ signaling depending on the cell type [44]. Lee et al. [45] added that CD133 promotes axon regeneration by down-regulating cholesterol synthesis via Smad signaling. Another pathway involved in CD133 is ERK, where ERK has been shown to increase the motility, invasion and metastasis of cancer cells [46]. The ERK and Smad signaling pathways are known to be pathways in which PAQR6 and ITPKB are involved. Based on research evidence and the results of literature studies that have been described previously, a common pathway found from the proposed biomarkers could be obtained, mainly in the PI3K/Akt, MAPK, Smad, Ras/Raf/MEK/ERK signaling pathways in GBM resistance which supports the process of GSC population growth as an adaptation response. and survival in cancer cells exposed to sustained exposure to DNA damaging substances.

In conclusion, this study analyzes that there is a link between GBM resistance genes and the origin of GBM through activation of the PI3K/Akt, MAPK, Smad, Ras/Raf/MEK/ERK signaling pathways.

Author Contribution Statement

All authors contributed equally in this study.

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Approval

This paper is an original article presenting the findings derived from a master's degree thesis entitled "In Silico and In Vitro Study of mRNA Biomarkers for Glioblastoma Multiforme Resistance to Temozolomide (TMZ): The Association with Stemness".

Data availability statement

All relevant data is presented within the manuscript. All sources also have been cited.

Ethical Approval

This research does not have an ethical document because the primary study was conducted in silico, utilizing computational models, and involved in vitro tests using established cell lines. Since no human participants or live animals were involved in the experimental process, ethical approval was deemed unnecessary. Both in silico methods and cell line-based assays generally fall outside the scope of formal ethical review, as they do not directly involve living subjects.

Conflict of Interest

The authors confirm that they have no conflicts of interest to disclose.

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