Expression of TMEM59L associated with radiosensitive in glioblastoma

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Research Article

Keywords: radiotherapy, radiosensitive, glioblastoma, TMEM59L

Posted Date: June 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1750404/v1

Abstract

Background:

Radiotherapy (RT) is one of the cornerstone of the glioblastoma (GBM) treatment paradigm. RT can significantly improve the prognosis of GBM; however, the resistance of tumor cells to radiation results in poor survival. The mechanism of radioresistance has not been fully elucidated.

Methods

The differentially expressed genes were screened based on RNA sequencing dataset of 226 primary glioblastomas and 134 recurrent glioblastomas that have undergone radiotherapy, combined with 15 pairs of primary and recurrent GBM obtained from the Chinese Glioma Genome Atlas. Real-time quantitative PCR was performed in three irradiated GBM cell lines（U87, LN229 and U251）to verify the result. The selected gene was investigated to the relationship between mRNA levels and clinical characteristics in the CGGA and TCGA dataset. Kaplan-Meier survival analysis and Cox regression analysis were used for survival analysis. Gene ontology (GO) and KEGG pathway analysis was used for bioinformatics analysis.

Results:

TMEM59L expression was significantly elevated in recurrent GBM who have received radiotherapy. The result was validated in three irradiated GBM cells. The high-expression of TMEM59L was enriched in *IDH* mutant and MGMT methylated gliomas and associated with a better prognosis. Gene ontology and KEGG pathway analysis demonstrated that TMEM59L was closely associated with cell proliferation, apoptosis, drug response, and DNA damage.

Conclusion

TMEM59L expression was increased after irradiation and resulted in longer survival. We speculated that the high expression of TMEM59L might affect radiotherapy sensitivity by contributing to reactive oxygen species and DNA damage repair.

1. Introduction

Glioblastoma (GBM) is the most common and lethal tumor of the central nervous system[1,2]. There is level 1 evidence that RT provided a clear survival benefit, which has been proved by numerous randomized controlled trials. However, despite elevated radiation dose and the improvement of RT equipment, the survival of GBM has not been significantly improved over the last 30 years. The resistance to the cytotoxic effects of RT is increasingly recognized as a significant impediment to effective radiotherapy. Currently, the median survival of glioblastoma is still less than 15 months.[3] It is urgently needed to identify reliable molecular targets and improve the effect of radiotherapy.

Radiotherapy potently induces massive cell death by triggering the activation of death signaling in cancer cells by directly or indirectly DNA damage [4,5]. However, a small portion of cancer cells may survive by activating compensatory survival signaling involving damage-repair signaling and reactive oxygen species (ROS) scavenging. Cancer cells that survived after radiotherapy could exhibit radioresistance and quickly tumor recurrence within the radiation field, which was clinically the most common recurrence pattern. We assumed that the differentially expressed genes after radiotherapy might be the key factor to elucidate the molecular mechanism of radioresistance.

In this study, we found that transmembrane protein 59L (TMEM59L) mRNA expression was up-regulated in recurrent GBM, which have received RT. The data was validated in three irradiated GBM cells. More importantly, the high expression of TMEM59L was significantly associated with longer survival in glioma patients. The informatics analysis found that TMEM59L is closely related to DNA damage and repair process. These findings indicated that TMEM59L might affect radiosensitive by regulating DNA damage and repair.

2. Materials And Methods

2.1.1 Clinical Samples

RNA sequencing dataset of 226 primary glioblastomas, 134 recurrent glioblastomas, combined with 15 pairs of primary and recurrent GBM obtained from Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/). The RNA-sequencing data and corresponding clinical information, including age, gender, histology, pathological subtype, MGMT promoter methylation， *IDH* status and survival information were downloaded from the CGGA database and The Cancer Genome Atlas (TCGA， http://cancergenome.nih.gov) as training cohort and validation cohort, respectively. This study was approved by the ethical review committee of the Beijing Tiantan Hospital（IRB KY2013-017-01）.

2.1 .2 Cell culture

Three human GBM cell lines, including U87, U251, and LN229 purchased from the Chinese Academy of Sciences Cell Bank were applied to cytological experiments in vitro. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Humidified incubators were supplied with an atmosphere containing 5% carbon dioxide at 37°C.

2.2.1 Cell irradiation

Irradiation methods were as follows: Precision X-ray Irradiator (PXi, America) was used. The irradiation field covered the culture dish, the distance from the source was 50 cm, and the dose rate was 180-200cGy/min. The radiation dose was given as three fractions of 36Gy with 12Gy for each fraction.

2.2.2 Real-time quantitative PCR

Total RNA was extracted after irradiation using a total RNA extraction kit (Tiangen China), following the manufacturer's instructions. The extracted RNA was used as a template to reverse transcription genomic RNA using a reverse transcription kit (Thermo, America). Quantitative PCR was conducted using SYBR Supermix Kit (Bio-Rad, America) to measure the mRNA expression of TMEM59L. The primer sequences for *TMEM59L* were the following: Forward, 5'- AGT CTC CCT ATG ACA GAG CCG -3', Reverse, 5'- GCT TCA CAC TCA GTT TGG GTG -3'. The post-irradiation time point of 0 4 8 hours was chosen for q-PCR according to extensive research on post-irradiation DNA damage and repair of oncology.

2.3 | Bioinformatics analysis

The correlation between TMEM59L mRNA expression and other genes were verified by Pearson's correlation analysis. The positive correlative gene ($r > 0.5$, $P < 0.05$) and the negative correlative gene ($r < 0.5$, $P < 0.05$) were selected for analysis. $P < 0.05$ was considered statistically significant. Gene ontology and KEGG pathway analysis were conducted in The Database for Annotation Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/)[6]

2.4 | Statistical analysis

All statistical analysis was conducted using SPSS 16.0 software, the R programming language 3.2.5 and the Graph Pad Prism 7.0 software. Student's *t-test* was used to compare differential expression after radiotherapy the different expression levels between grades or subtypes. The prognostic significance was assessed by Kaplan-Meier survival analysis and univariate as well as multivariate Cox regression analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1 TMEM59L mRNA expression was elevated after irradiation.

Student's t-test was performed on the CGGA database and matched data to identify differentially expressed genes after radiotherapy (Figure-1 A and B). TMEM59L expression was significantly elevated both in recurrent GBM, which have received RT ($P<0.001$) and in the matched patients ($P=0.04$). The result was validated in virto experiments that TMEM59L was significantly up-regulated in the three irradiated cell lines(U87 2.0-fold at 8 hours $P=0.04$, LN229 2.9-fold $p=0.019$ and U251 1.7-fold P 0.01) compared with their parent cells. The expression level gradually increased after irradiation between 0 to 8 hours (Figure-1 C, D and E).

3.2.1 The high expression of TMEM59L was associated with a better prognosis.

Kaplan–Meier survival curves were constructed to determine the prognosis value to evaluate if the TMEM59L expression is associated with survival in glioma patients. TMEM59L expression level was divided into low/high groups according to the median value of TMEM59L mRNA expression in 325 patients. The results showed that high expression of TMEM59L was related to longer overall survival

(OS) than low expression in the CGGA dataset (Figure-2). Multivariate Cox analyses showed that TMEM59L expression (HR: 0.667, 95% CI: 0.56-0.79, P 0.001) was an independent predictor of longer survival Figure-2 A, C. Similar results were validated in 699 patients from the TCGA dataset (Figure-2 B, D). The results suggested that TMEM59L might be a novel independent prognostic biomarker for glioma patients.

3.2.2 TMEM59L expression level shows a subtype preference

We further invested the clinical prognostic significance of the TMEM59L in glioma.

The high-expression of TMEM59L was enriched in *IDH* mutant and MGMT methylated glioma (Table-1, Figure-3). The TMEM59L expression level was negatively correlated with tumor grade Supplemental Figure-1 and significantly up-regulated in the favorable neural subtype.[7] On the contrary, TMEM59L expression was down-regulated in the mesenchymal type with poor prognosis[8]. The ROC curves showed that the area under the curve (AUC) was up to 80.2% and 89.6% in the CGGA and TCGA sequencing dataset, respectively Supplemental Figure-2.

Table 1 Clinical features of patients with glioma in CGGA and TCGA stratified by TMEM59L level

	TCGA(699)		CGGA(325)	
Characteristics	Low(349)	High(350)	Low(162)	High(163)
Gender				
Male	192	176	106	97
Female	135	133	56	66
NA	22	41	0	0
Age(years)				
≤40	101	145	57	86
40	226	164	105	77
Na	22	41	0	0
Grade				
2	76	147	27	76
3	110	135	36	43
4	141	27	99	44
Na	22	41	0	0
IDH mutation				
Mutant	158	285	62	114
Wild type	184	62	100	49
NA	7	3	0	0
MGMT-status				
Methylated	209	283	66	73
Unmethylated	109	59	75	42
NA	31	8	21	48

3.3 TMEM59L related biological process

Pearson correlation analysis was performed to investigate the biological process tightly associated with TMEM59L expression in the CGGA and TCGA sequencing datasets. Those genes tightly correlated with TMEM59L expression (Pearson $|R| > 0.5$ $P < 0.05$) in the CGGA and (Pearson $|R| > 0.4$, $P < 0.05$) in the TCGA were used for Gene ontology analysis with DAVID. Pearson correlation analysis was also applied to several classical immune checkpoints and GSCs related genes in CGGA and TCGA datasets.

It is shown that the negative correlation genes with TMEM59L expression are highly enriched in the immune and inflammatory response, cell proliferation and migration, apoptosis process, response to drug and DNA damage. The positive correlation genes tended to be enriched in biological processes that are normal and indispensable, such as neurotransmitter secretion and nervous system development. In addition, TMEM59L expression was negatively related to CD44, STAT3, IL6 and FUT4 and positively related to L1CAM（Supplemental Figure-3）. TMEM59L expression was tightly related to the PD1 family, B7 family, LAG3 , TIM3, CTIL4 and IDO regarding the immune system.

KEGG pathway analysis revealed that the negatively related genes with TMEM59L expression were enriched in the PI3K-AKT signaling pathway, the NF-kappa B, P53 signaling pathway and the positively related genes were enriched in the GABA ergic-synapas, Glutamatergic synapse. The two datasets shared all the results mentioned above （Figure-4）.

Discussion

In this study, we identified and verified the increased expression of TMEM59L after radiotherapy through a public database and in vitro experiment. Bioinformatics analysis revealed that TMEM59L was closely related to the DNA damage and repair process. Importantly, the TMEM59L expression showed great value in the prognosis of glioma patients. Through these results, we may infer that the high expression of TMEM59L could enhance tumor radiosensitivity. These findings provide an important reference for the study of GBM radiosensitivity.

TMEM59L is a newly identified brain-specific membrane-anchored protein that belongs to a large family of genes encoding transmembrane (TMEM) proteins [9,10]. Many TMEMs function as channels to permit the transport of specific substances across the biological membrane and fulfill important physiological functions such as mediating cell chemotaxis, inflammatory signaling pathways, apoptosis, autophagy, etc[11-14]. Recently, several TMEM proteins have been revealed to act as oncogenes or tumor suppressors in a tumor. Moreover, TMEMs could also impact chemoresistance by exerting an anti-apoptotic function. For example, TMEM7 was reported that play a tumor suppressor role in hepatocellular carcinoma. TMEM71 has been reported to act as an oncogene in GBM and may indicate poor sensitivity to TMZ therapy[15]. Zhang has reported that TMEM59L can mediate oxidative stress-induced cell death through autophagy and apoptosis pathway. Down-regulation of TMEM59L protects neurons against oxidative stress[16]. However, the function and expression characteristics of TMEM59L has not been studied in malignant tumor and radiotherapy.

Our research demonstrated that the expression of TMEM59L showed predictive value for prognosis in glioma and intimately related to the DNA damage and repair process. It is well established that radiation executes anti-tumor DNA-damaging effects through directly or indirectly DNA damage. About two-thirds of DNA damage ascribing to radiotherapy is caused by indirect effects via the generation of ROS. Interestingly, the high-expression of TMEM59L has concentrated in *IDH* mutation-type glioma. Several studies have highlighted the important role of *IDH* in defense against radiation-induced

oxidative injury.[17] The *IDH* mutation could change the metabolic state of the cell and increase the oxidation of NADPH, which renders the affected glioblastoma cells more vulnerable to ROS induced by irradiation. Previous studies have reported that the *IDH*1 mutation makes cells more vulnerable to radiation[18,19]. In addition, the correlation analysis displayed TMEM59L showing well consistency with two GCS markers in radiosensitivity. TMEM59L expression was negatively related to CD44, which was reported as radioresistance markers and positively related to L1CAM, which was correlated inversely with radioresistance. All these results indicated that the high-expression of TMEM59L might increase radiosensitive though down-regulate ROS scavenge.[20,21]

Radiation-induced damages could also trigger a large network of intracellular signaling events. Multiple studies have reported that the expression of the PI3K/Akt signaling pathway could stimulate the DNA damage response through the regulation of proteins and failure of chemoradiotherapy by increasing repair of damaged DNA [22]. Importantly, several studies have proven that targeting the PI3K/Akt pathway by specific repressors in association with radiation appears to enhance radiosensitization[23,24].NF-κB was found to be activated and associated with a higher grade of astrocytic tumors.[25] The activation of NF-κB could occur in response to DNA damaging agents and provoke multiple radioresistance signals, which attenuate the lethal effects of radiation. Moreover, inhibition of NF-κB was proved to be an effective strategy to enhance tumor radiosensitivity.[26,27] Our result showed that TMEM59L expression was negatively related to these pathways. It can be inferred that TMEM59L might affect radiosensitivity by affecting PI3K/Akt and NF-κB inhibition.

Regarding the immune system, the expression of immune checkpoint molecules in the tumor microenvironment could affect the efficiency of the immune response. Evidence accumulated over recent years has revealed that exposure of cancer cells to radiation may cause upregulation of PD-L1, leading to resistance to radiotherapy. Abrogation of both CTLA-4 and PD-1 pathways has been associated with higher radiosensitivity[28]. Our research demonstrated that the expression of TMEM59L was negatively correlated with immune checkpoints. The results indicate that up-regulated TMEM59L expression might be important in maintaining immune response activity.

We found the effect of TMEM59L on glioma prognosis and radiotherapy effect in gene function and correlation analysis; however, the specific mechanism is still unclear. A more deep study of signaling pathways and interacting genes would focus on further research.

In conclusion，the increased expression of TMEM59L after irradiation might affect radiotherapy sensitivity through regulating reactive oxygen species and DNA damage repair. Further research may help delineate processes that contribute to the radiosensitive and improve the efficacy of radiotherapy.

Declarations

Funding

This work was supported by The National Natural Science Foundation of China (Grant Numbers: 82001778), The Capital Medical Development Research Fund (Grant Numbers: 2020-2-1072).A multicenter study of radiosurgery for nonfunctional pituitary adenomas. (Grant Numbers: 2019-N-11-35)

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Beijing Tiantan Hospital（IRB KY2013-017-01）

Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflict of interest.

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Figures

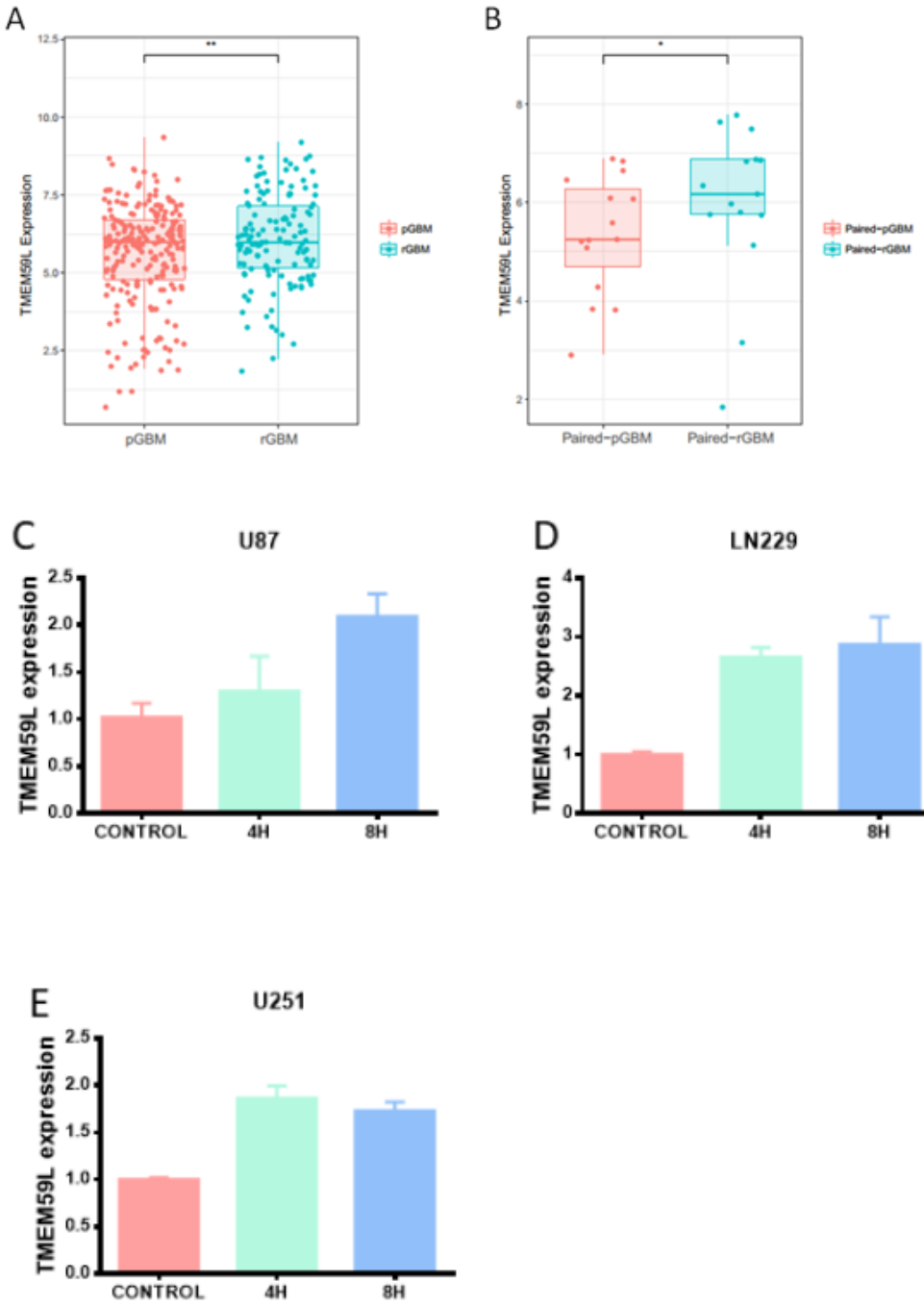

Figure 1

(A) The different gene between primary GBM and recurrent GBM in CGGA.

(B) The different gene between primary GBM and recurrent GBM in matched dataset.

(C-E)TMEM59L expression in U87, LN229 and U251cell line at 0 hour, 4 hour and 8 hours after irradiated.

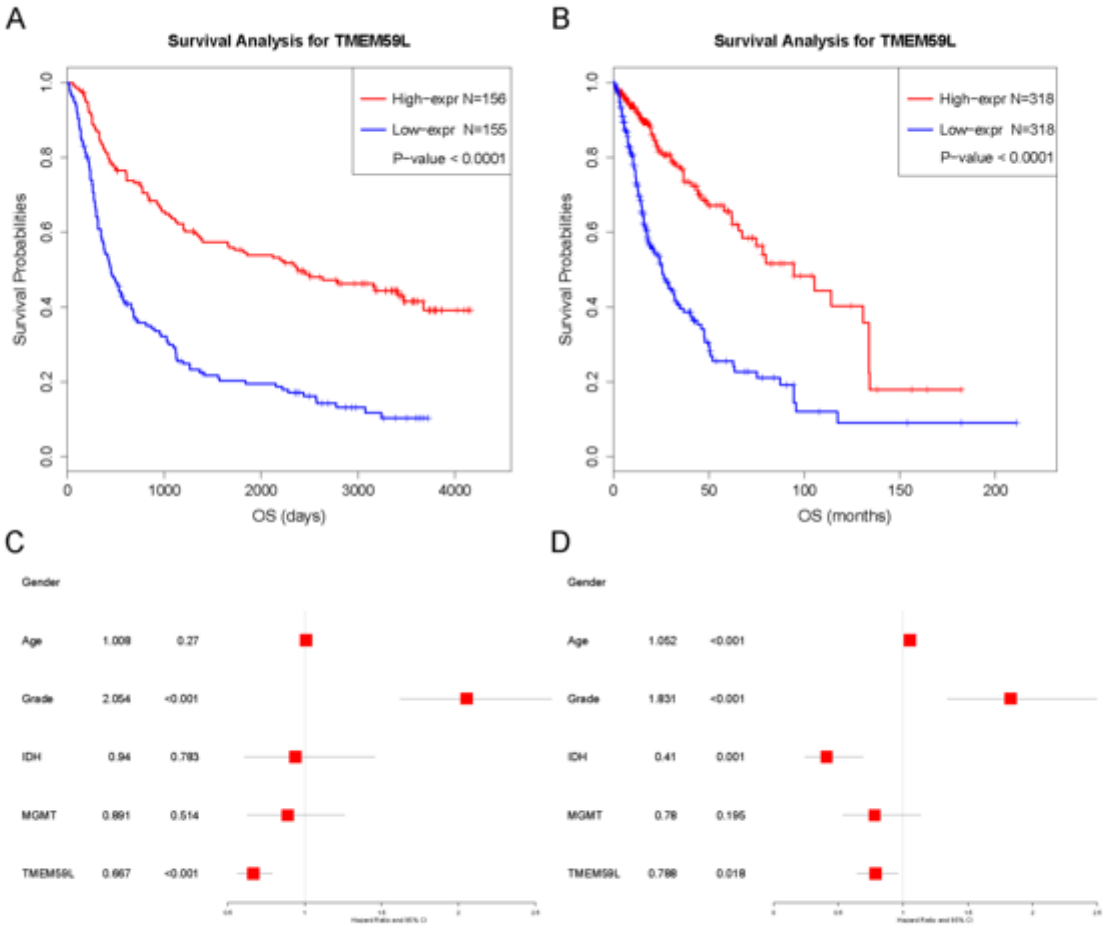

Figure 2

(A-B) K-M analysis in glioma from the CGGA and TCGA databases.

(C-D) Forest plot in CGGA and TCGA dataset.

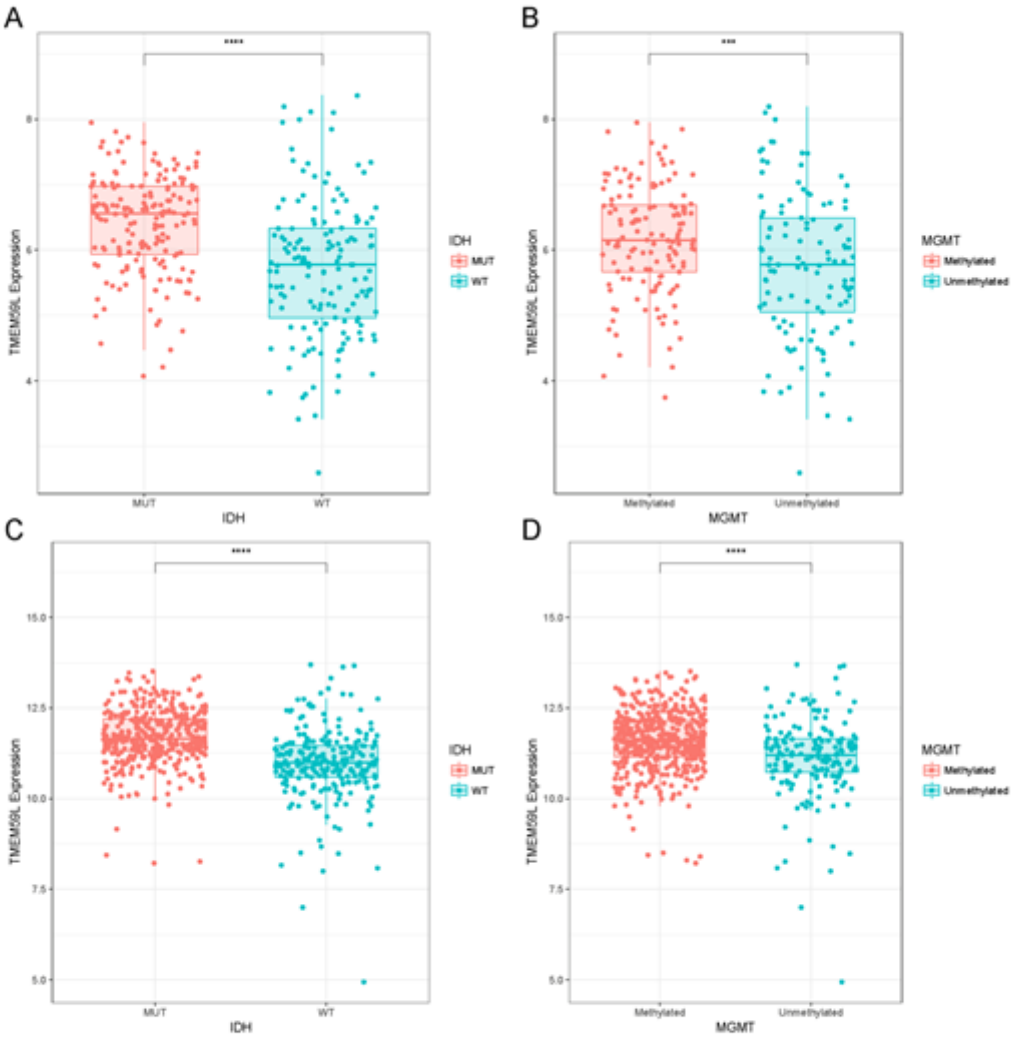

Figure 3

(A,C) TMEM59L expression with different IDH status in CGGA and TCGA databases.(B,D) TMEM59L expression according to MGMT status in CGGA and TCGA databases. ****P < 0.001,, ***P < 0.05

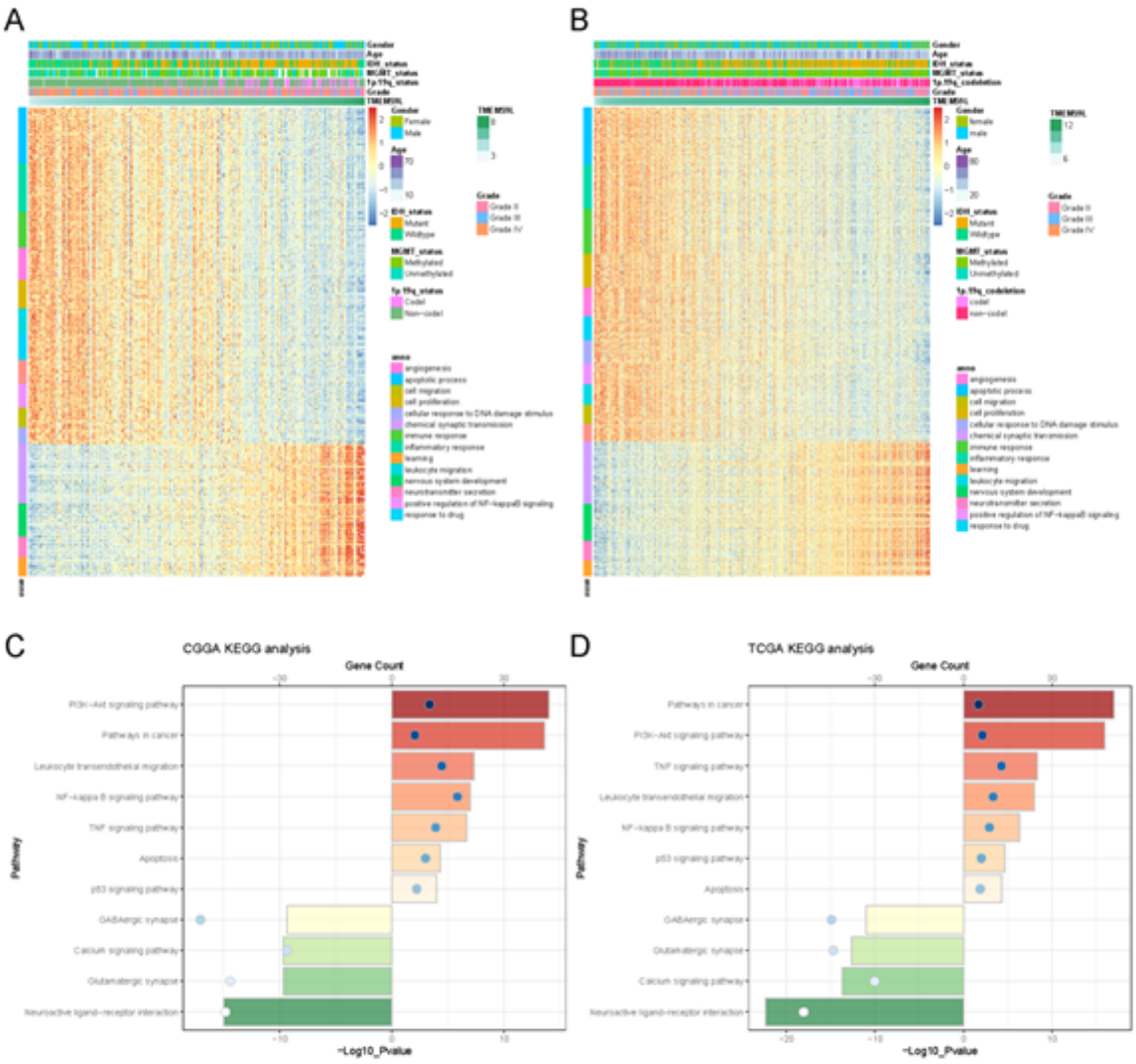

Figure 4

Biological function and pathway analysis in CGGA and TCGA datasets.

(A-B) Gene ontology analysis of TMEM59L expression in glioma. The samples were ranked according to TMEM59L expression from low to high.

(C-D) KEGG analysis of TMEM59L xpression in glioma. The bar charts represented the count and the circle represented the P value.

Supplementary Files

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- SupplementaryFigures.docx