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*Original Article*

# Expression and clinical significance of *TIMELESS* in glioma

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**Abstract:** In recent years, studies have shown that *TIMELESS*, as an oncogene, is involved in progression of cancers. However, its relationship with prognosis in glioma patients is rarely reported. Our purpose was to explore the role of *TIMELESS* in glioma. Based on 1814 glioma samples from multiple databases such as The Cancer Genome Atlas (TCGA), The Chinese Glioma Genome Atlas (CGGA), and The Gene Expression Omnibus (GEO), we use a variety of bioinformatics methods to verify the mechanism of action of *TIMELESS* in glioma from mRNA to protein, from appearance to mechanism analysis, from clinical features to prognosis. Then, the connectivity map (CMap) tool was used to predict drugs that inhibit the expression of *TIMELESS*. First, we found *TIMELESS* is highly expressed in glioma at mRNA and protein levels. Second, *TIMELESS* is an independent risk factor in prognosis and has suitable clinical diagnostic value in glioma. It was also positively correlated with World Health Organization (WHO) grade, age, and histology, and negatively correlated with *isocitrate dehydrogenase (IDH) 1* mutation and *1p19q* codeletion. Third, base excision, cell cycle, and mismatch repair pathway were activated by *TIMELESS* in glioma. We predict small molecules to inhibit *TIMELESS* such as 8-azaguanine, gw8510, 6-thioguanosine, and ursodeoxycholic acid. This study is the first comprehensive analysis of *TIMELESS*, revealing a relationship between this novel oncogene, clinical characteristics of patients with glioma, and a mechanism leading to poor prognosis. It also provides a biomarker for diagnosis and treatment of glioma and reveal the pathologic progress of glioma at the genetic level.

**Keywords:** Glioma, *TIMELESS*, biomarker, oncology, small molecule compound

## Introduction

Glioma is a primary malignant tumor derived from neuroepithelial cells [1]. Its annual incidence rate is 1.6% of systemic tumors, but its mortality is 2.5% of systemic tumors [2]. Computed tomography and magnetic resonance imaging are currently the main adjuncts for the diagnosis of glioma [3, 4]. Patients with glioma in the middle and late stage often have typical imaging changes and a series of dysfunctions [4]. Surgical treatment and adjuvant radio-chemotherapy are the main means of clinical treatment. However, most patients after treatment have neurological dysfunction, and poor prognosis brings huge economic and psychologic burdens to society and families [5]. With the remarkable effect of chimeric antigen receptor T-cell immunotherapy in treating leukemia, molecular targeted therapy has rekindled hope [6]. Therefore, in order to improve the treatment of glioma patients, it is urgent to find new effective molecular markers.

At present, special molecular markers discovered by scientists have been widely used in the early diagnosis of glioma and personalized treatment. For instance, with *IDH1*, in low-grade glioma, the prognosis of *IDH* mutant is better than that of wild type [7]. Next, $O_6$-methylguanine-DNA methyltransferase (MGMT) can promote the successful escape of tumor cells

from alkylated chemotherapeutic drugs, resulting in patients being insensitive to drugs and having poor therapeutic effect [8]. In addition, epidermal growth factor receptor (EGFR) participates in the formation of tumors through various channels, often related to the prognosis of glioblastoma [9, 10]. However, due to the diversity of glioma formation and the complexity of the tumor microenvironment, existing theories do not fully reveal the specific pathogenesis of glioma [11]. Therefore, discovery of new molecular markers will help to reveal the mechanism and provide molecular targets for prognosis and discovering therapeutic drugs.

Cytogenetic and molecular genetic studies have shown that tumor development is a complex process with multiple factors and stages [12, 13]. Genomic instability is considered a driving force in all stages of tumorigenesis [14]. *TIMELESS* participates in the maintenance of genome stability through multiple channels. For example, Anthony et al. found that *TIMELESS* can compile a conserved protein and gather it in the replication site of the nucleus, thereby contributing to DNA replication efficiency by combining Tipin [15]. Lauren and Si et al. found that *TIMELESS* migrated to DNA loss sites in a PARP1-dependent manner, and participated in the DNA repair process, and homologous recombination [16, 17]. Further research found that *TIMELESS* can also promote the malignant behavior and poor prognosis of tumors. *TIMELESS* promotes the insensitivity of nasopharyngeal carcinoma to cisplatin through Wnt/β-catenin signaling pathway, thereby promoting tumor epithelial cell metastasis [18]. *TIMELESS* overexpression promotes the proliferation of colorectal cancer cells [19]. Qiu et al. found that overexpression of *TIMELESS* correlates with poor prognosis in kidney and liver cancer [20]. However, there is little research about the role and mechanism of *TIMELESS* in glioma.

In this study, large samples from multiple databases were used for the first time to find a correlation between the expression of Timeless and clinical features, and further explore the molecular mechanism of Timeless involvement in tumor formation and development. We strongly believe that *TIMELESS* can be used as a molecular marker for clinical diagnosis and treatment and provides a new direction for revealing the pathological mechanism of glioma.

## Materials and methods

### *Data sources*

GEPIA (http://gepia.cancer-pku.cn/) is a well-known online tool for visual analysis of TCGA and GTEx data. Because of its simple interface, simple operation, and effective analysis, it is popular with researchers. Our study investigated the expression level of *TIMELESS* in various human tumor tissues and corresponding normal samples from this database. These include 163 GBM samples, 518 LGG samples, and 207 normal brain tissues.

CGGA (http://www.cgga.org.cn/) is an open database that has been published for several years, is especially used for glioma research, and provides a platform for researchers to analyze clinical information, data expression, gene copy number, and methylation [21]. Apart from missing data such as survival time, gender, and so on, our study obtained the RNA-seq of 748 glioma tissue samples and mRNA chip data of 268 glioma tissue samples, for further analysis and processing. The filtered information is shown in Tables S1 and S2.

TCGA (http://cancergenome.nih.gov/) is a landmark public free database of cancer genomics, which contains many types of data such as genome, epigenome, transcriptome, and proteome. It has improved our ability to diagnose, treat, and prevent cancer and is made available to anyone in the research community [22]. After the deletion of missing clinical information data, we finally collected information on 653 tumor samples, see Table S3.

GEO (https://www.ncbi.nlm.nih.gov/geo/) is an open database serving the public, characterized by containing gene sequencing and chip data of multiple organisms based on multiple platforms [23]. It can provide a continuous data set so that researchers can systematically study the direction of interest. GSE4290 included 77 glioma and 23 normal brain tissue samples; GSE116520 included 34 glioma and 8 normal brain tissue samples; and GSE50161 included 34 glioma and 13 normal brain tissue samples. We use the limma package in the R (version 3.6.1) language to analyze *TIMELESS* expression in glioma tissue and normal brain tissue [24] (Fold Change value of (|log2FC|) >1 and $p$-value <0.05).

The Human Protein Atlas (HPA) (http://www.proteinatlas.org/) is an open and free web tool that contains high-resolution images of millions of normal human tissues, cancer tissues, and human cell line proteins [25]. In order to obtain the differences in *TIMELESS* protein levels, we downloaded the *TIMELESS* immunohistochemical results from the database and divided them into a normal group, high-grade glioma group, and low-grade glioma group.

*Gene set enrichment analysis*

Gene Set Enrichment Analysis (GSEA) is used to evaluate the distribution trend of genes in a pre-defined gene set in the gene table sorted by phenotype correlation, so as to judge their contribution to phenotype [26]. We first standardized the processing of the original data from multiple databases (CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq), and then divided the genes into two groups (high expression group, low expression group) according to the level of *TIMELESS*. GSEA 4.0.2 jar software was used in this study. The number of permutations was adjusted to 1000, and the genome database was changed to the KEGG cell signaling pathway.

*CMap*

CMap (https://cmap.ihmc.us/) helps us understand human diseases and accelerate the discovery of new therapies by creating and analyzing large perturbation data sets. We first used correlation analysis to obtain 10 genes positively related to *TIMELESS* and 10 genes negatively related. Then, we used the GPL570 platform to convert related genes into probe information, and then uploaded it to the CMap tool to analyze potential inhibitors of *TIMELESS* ($P$<0.05 and enrichment <-0.88).

*Statistical analysis*

The original data in this study were analyzed by Perl and R software. Wilcoxon test was used to assess the expression of *TIMELESS* between tumor tissue and non-tumor tissue. We used the R software to calculate the relationship between *TIMELESS* and the survival time of glioma patients through the Cox regression and Kaplan-Meier method and drew a survival curve. Then, univariate and multivariate analysis revealed whether high expression of *TIMELESS* was an independent factor for patients' poor prognosis. Cox regression evaluated the value of *TIMELESS* as an independent factor. We discuss the relationship between clinical data and *TIMELESS* expression in glioma by using Wilcoxon or Kruskal-Wallis test.

## Results

*Abnormally high expression of TIMELESS in glioma*

First, we analyzed the expression of *TIMELESS* in a variety of tumors and corresponding normal tissues through the GEPIA tool, and found that *TIMELESS* had high expression in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), glioblastoma multiforme (GBM), brain lower grade glioma (LGG), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS), but had low expression in acute myeloid leukemia (AML) (**Figure 1A**).

To further study the mechanism of *TIMELESS* in tumors, we chose glioma. Through GSE4290 (77 glioma tissue samples and 23 normal brain tissue samples), GSE50161 (34 glioma tissue samples and 13 normal brain tissue samples), and GSE11652 (34 glioma tissue samples and 8 normal brain tissue samples) put through database analysis, we again verified that *TIMELESS* was highly expressed in glioma (**Figure 1B-D**, $P$<0.001). Through the mutual verification by multiple data sets, we could effectively improve the credibility of our results. This study will lay a foundation for further study of the mechanism of *TIMELESS* in glioma.

*High expression of TIMELESS is an unfavorable factor for prognosis of glioma*

In order to evaluate the relationship between the high expression of *TIMELESS* and prognosis of glioma patients, the original data from the CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq databases were divided into a high expression group and a low expression

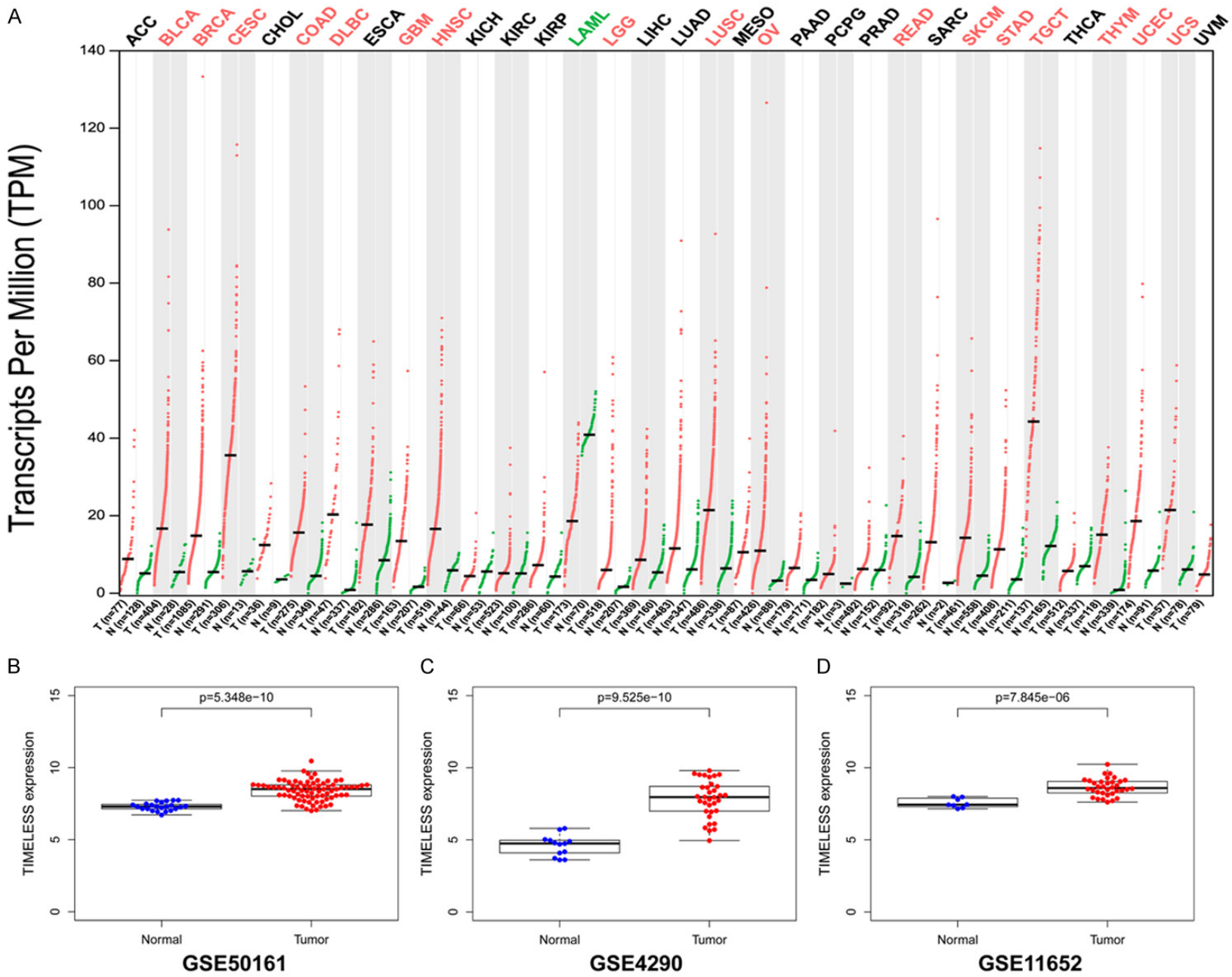

A
ACC BLCA BRCA CESC CHOL COAD DLBC ESCA GBM HNSC KICH KIRC KIRP LAML LGG LIHC LUAD LUSC MESO OV PAAD PCPG PRAD READ SARC SKCM STAD TGCT THCA THYM UCEC UCS UVM
Transcripts Per Million (TPM)
0 20 40 60 80 100 120 140
B
p=5.348e−10
TIMELESS expression
Normal
Tumor
GSE50161
C
p=9.525e−10
TIMELESS expression
Normal
Tumor
GSE4290
D
p=7.845e−06
TIMELESS expression
Normal
Tumor
GSE11652

**Figure 1.** Expression level changes of *TIMELESS* in glioma. A. *TIMELESS* is widely expressed in a variety of tumors: red indicates cancer with up-regulation of *TIMELESS*, while green indicates a decrease in the expression of *TIMELESS* in malignant tissues. B-D. *TIMELESS* expression is higher in glioma than normal brain tissues from GEO (GSE4290, GSE50161, GSE116520, $P<0.001$).

group according to the expression of *TIMELESS*. First, compared with the low expression group of *TIMELESS* in 376 glioma patients through the Kaplan-Meier method, the high expression of *TIMELESS* in 372 glioma patients showed a survival time that was significantly less than in the low expression group based on CGGA RNA-seq (**Figure 2A**). Second, compared with the low expression group in 134 glioma patients through the Kaplan-Meier method, the high expression of *TIMELESS* in 134 glioma patients showed that *TIMELESS* can also reduce the survival time of glioma patients based on the CGGA microarray (**Figure 2B**). Finally, in order to test the effect high *TIMELESS* expression in different ethnic groups on prognosis, we further verified this by using TCGA RNA-seq: this dataset mainly contains American race, whereas CGGA database contains data from Chinese race. It was found that the increased expression level of *TIMELESS* could significantly reduce the overall survival of 653 glioma patients (**Figure 2C**). The results were surprisingly consistent with the CGGA database. However, whether high expression of *TIMELESS* is an independent factor for poor prognosis requires further study.

*TIMELESS high expression has prognostic value*

To explore whether the high expression of *TIMELESS* has value for the prognosis of glioma, we analyzed the above three sets of data and by Cox regression. The high expression of *TIMELESS* was an independent factor for glioma patients with a poor prognosis and had diagnostic value at 1 year, 3 years, and 5 years from CGGA RNA-seq (**Figure 2D**), CGGA microarray (**Figure 2E**), and TCGA RNA-seq (**Figure 2F**). The results suggest that *TIMELESS* can be used as a biomarker for the diagnosis and individualized treatment of glioma.

*High expression of TIMELESS is an independent factor for poor prognosis of glioma*

We used univariate and multivariate analysis to analyze the relationship between clinical sample information and the survival status of glioma patients. From the analysis of the common results in the above three databases, we found that *TIMELESS* expression and glioma grade were closely related to the prognosis of glioma patients (**Figure 3A-F**). Detailed results are as follows: by univariate analysis, *TIMELESS* expression and patient survival time had significant statistical significance in CGGA RNA-seq (HR: 1.733, 95% CI: 1.587-1.892, $P<0.001$, **Figure 3A**), CGGA microarray (HR: 1.902, 95% CI: 1.636-2.212, $P<0.001$, **Figure 3C**) and TCGA RNA-seq (HR: 1.087, 95% CI: 1.064-1.109, $P<0.001$, **Figure 3E**). By multivariate analysis, it also has statistical significance in CGGA RNA-seq (HR: 1.252, 95% CI: 1.137-1.379, $P<0.001$, **Figure 3B**), CGGA microarray (HR: 1.483, 95% CI: 1.234-1.783, $P<0.001$, **Figure 3D**), and TCGA RNA-seq (HR: 1.034, 95% CI: 1.004-1.065, $P<0.05$, **Figure 3F**). At the same time, by univariate analysis, glioma grade and patient survival time had significant statistical significance in CGGA RNA-seq (HR: 2.883, 95% CI: 2.526-3.291, $P<0.001$, **Figure 3A**), CGGA microarray (HR: 2.567, 95% CI: 2.125-3.100, $P<0.001$, **Figure 3C**), and TCGA RNA-seq (HR: 4.634, 95% CI: 3.727-5.760, $P<0.001$, **Figure 3E**). By multivariate analysis, it also had statistical significance in CGGA RNA-seq (HR: 2.506, 95% CI: 1.825-3.441, $P<0.001$, **Figure 3B**), CGGA microarray (HR: 2.402, 95% CI: 1.389-4.155, $P<0.005$, **Figure 3D**) and TCGA RNA-seq (HR: 3.044, 95% CI: 2.401-3.860, $P<0.001$, **Figure 3F**). But, due to incomplete clinical data in the TCGA RNA-seq database, HR value of the age crossed the invalid line in the CGGA microarray multivariate analysis. Thus, whether post-resuscitation syndrome (PRS) type and age are an independent factor for glioma patients requires further analysis. In conclusion, *TIMELESS* high expression is an independent factor for poor prognosis in glioma.

*Relationship between TIMELESS and clinical features of poor prognosis in glioma*

The results of Wilcoxon or Kruskal-Wallis test correlation analysis showed that abnormally high expression of *TIMELESS* was positively

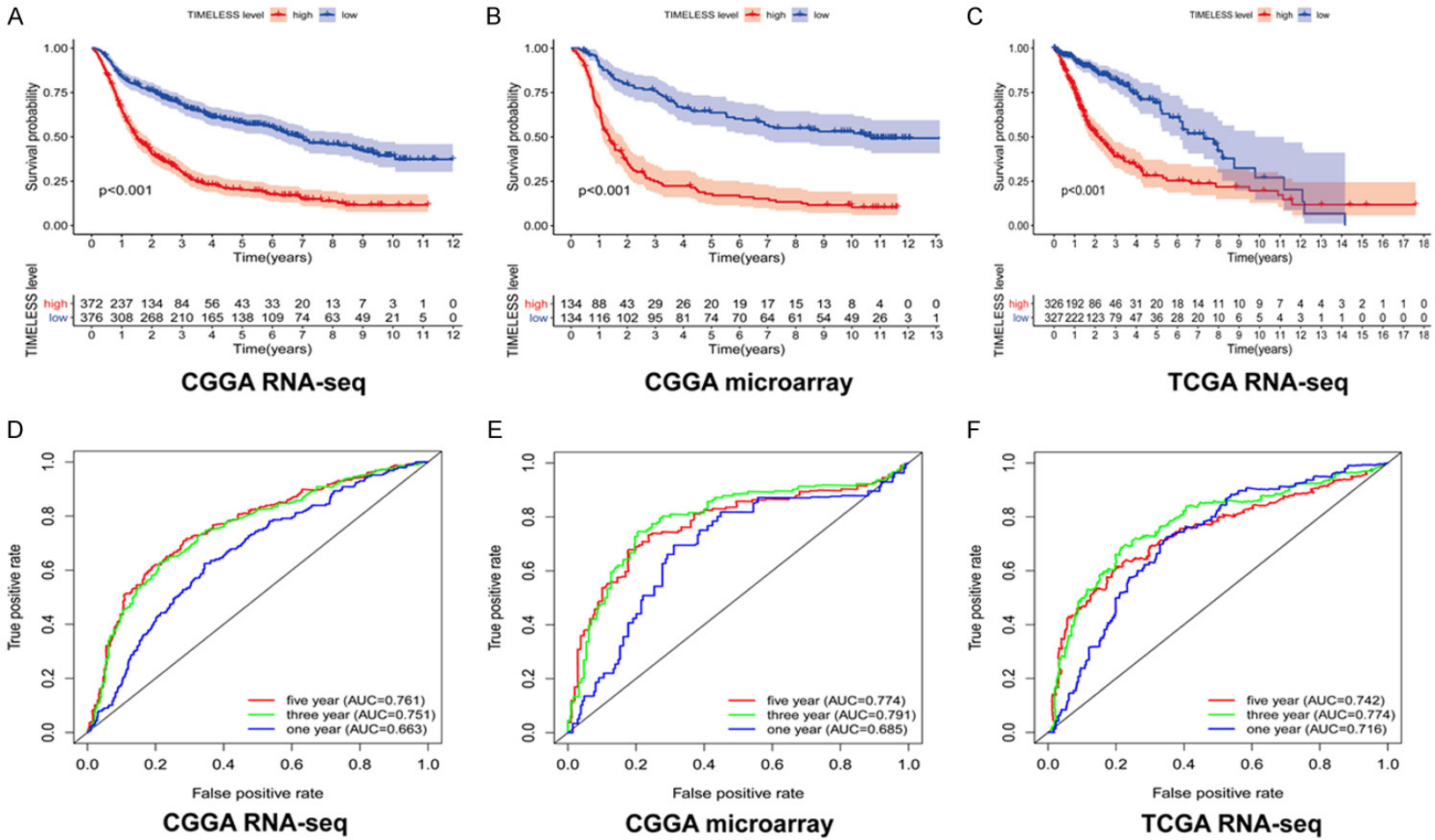
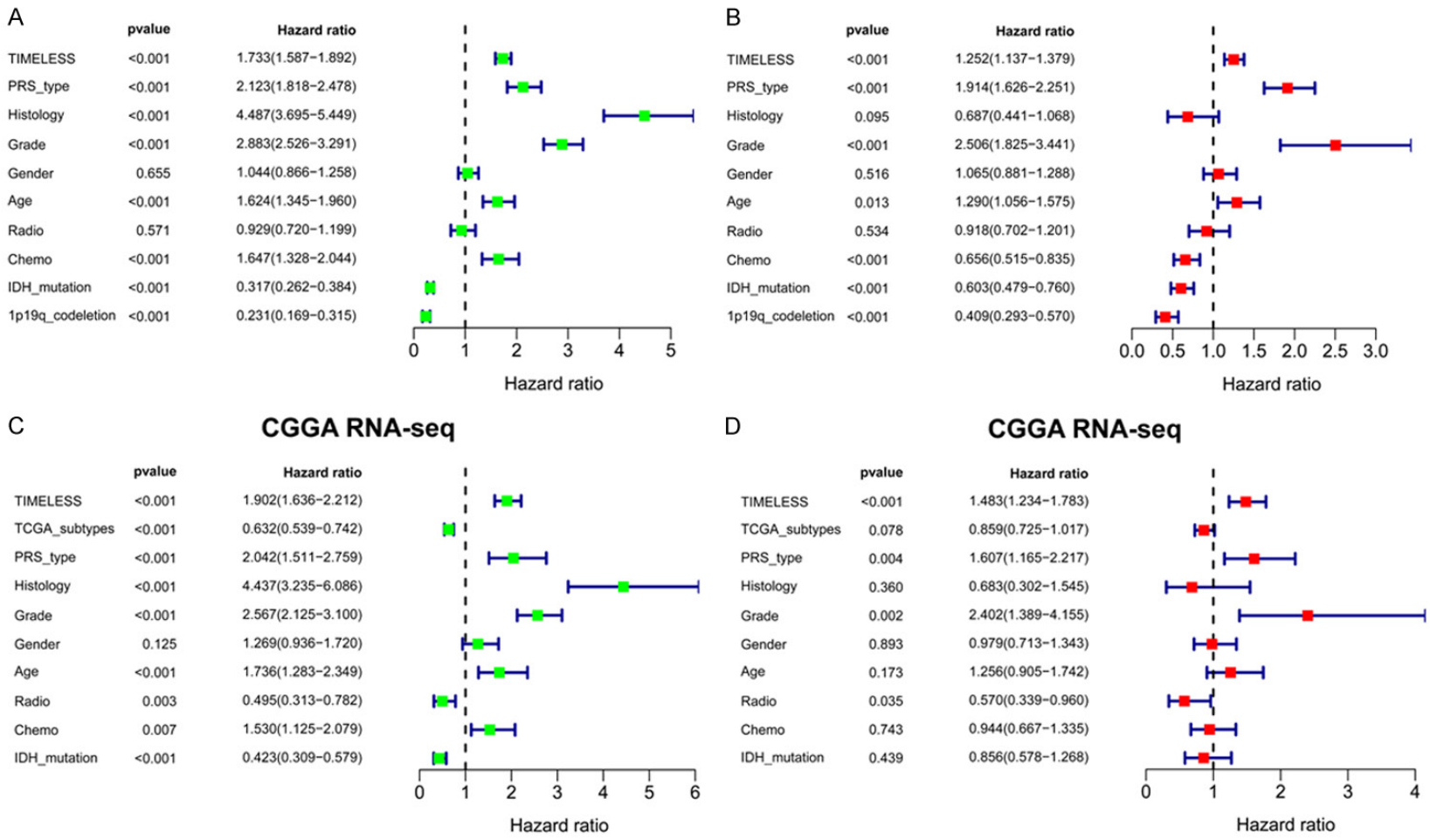



**Figure 2.** Correlation analysis of *TIMELESS* and survival time of patients based on multiple databases. A, D. In CGGA RNA-seq; B, E. In CGGA microarray. C, F. In TCGA RNA-seq. P<0.05.


A
pvalue
Hazard ratio
TIMELESS <0.001 1.733(1.587−1.892)
PRS_type <0.001 2.123(1.818−2.478)
Histology <0.001 4.487(3.695−5.449)
Grade <0.001 2.883(2.526−3.291)
Gender 0.655 1.044(0.866−1.258)
Age <0.001 1.624(1.345−1.960)
Radio 0.571 0.929(0.720−1.199)
Chemo <0.001 1.647(1.328−2.044)
IDH_mutation <0.001 0.317(0.262−0.384)
1p19q_codeletion <0.001 0.231(0.169−0.315)
0 1 2 3 4 5
Hazard ratio
B
pvalue
Hazard ratio
TIMELESS <0.001 1.252(1.137−1.379)
PRS_type <0.001 1.914(1.626−2.251)
Histology 0.095 0.687(0.441−1.068)
Grade <0.001 2.506(1.825−3.441)
Gender 0.516 1.065(0.881−1.288)
Age 0.013 1.290(1.056−1.575)
Radio 0.534 0.918(0.702−1.201)
Chemo <0.001 0.656(0.515−0.835)
IDH_mutation <0.001 0.603(0.479−0.760)
1p19q_codeletion <0.001 0.409(0.293−0.570)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
Hazard ratio
C
CGGA RNA-seq
pvalue
Hazard ratio
TIMELESS <0.001 1.902(1.636−2.212)
TCGA_subtypes <0.001 0.632(0.539−0.742)
PRS_type <0.001 2.042(1.511−2.759)
Histology <0.001 4.437(3.235−6.086)
Grade <0.001 2.567(2.125−3.100)
Gender 0.125 1.269(0.936−1.720)
Age <0.001 1.736(1.283−2.349)
Radio 0.003 0.495(0.313−0.782)
Chemo 0.007 1.530(1.125−2.079)
IDH_mutation <0.001 0.423(0.309−0.579)
0 1 2 3 4 5 6
Hazard ratio
D
CGGA RNA-seq
pvalue
Hazard ratio
TIMELESS <0.001 1.483(1.234−1.783)
TCGA_subtypes 0.078 0.859(0.725−1.017)
PRS_type 0.004 1.607(1.165−2.217)
Histology 0.360 0.683(0.302−1.545)
Grade 0.002 2.402(1.389−4.155)
Gender 0.893 0.979(0.713−1.343)
Age 0.173 1.256(0.905−1.742)
Radio 0.035 0.570(0.339−0.960)
Chemo 0.743 0.944(0.667−1.335)
IDH_mutation 0.439 0.856(0.578−1.268)
0 1 2 3 4
Hazard ratio

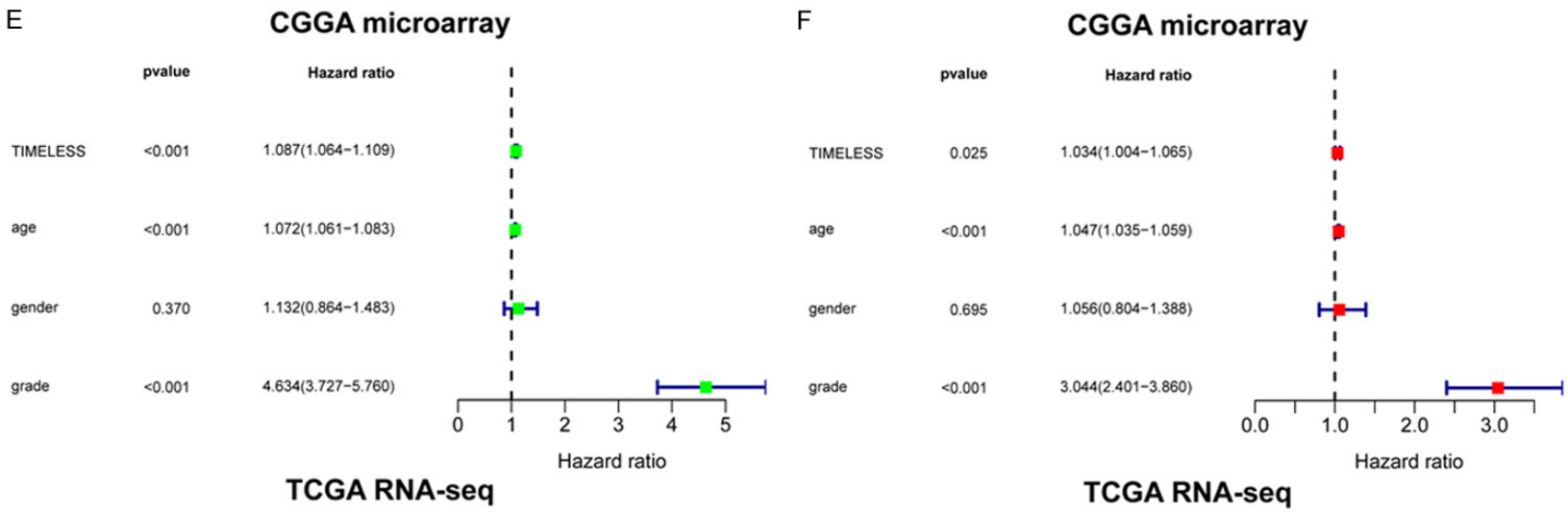



**Figure 3.** *TIMELESS* is an independent factor for poor prognosis in glioma by univariate and multivariate analysis. A, C, E. Univariate analysis from CGGA RNA-seq, CGGA microarray and TCGA RNA-seq. B, D, F. Multivariate analysis from CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq.

correlated with WHO grade, PRS type, chemo status, and histology (**Figure 4A**, **4C**, **4D**, **4G**, $P<0.001$), but *IDH1* mutation and *1p19q* codeletion states were negatively correlated (**Figure 4E**, **4F**, $P<0.001$) in the data of CGGA RNA-seq. It was positively correlated with WHO grade and histology, and negatively correlated with IDH1 mutation (**Figure 4A**, **4E**, **4G**, $P<0.001$) in CGGA microarray. Finally, it also was positively correlated with WHO grade and age in the data of TCGA RNA-seq (**Figure 4A**, **4B**, $P<0.001$). By analyzing the above three databases, we found that low expression of *TIMELESS* was closely related to low tumor grade, *1p19q* joint deletion, and *IDH1* mutation. As clinicaldata show, *1p19q* joint deletion, and *IDH1* mutations often occur in low-grade glioma, and patients often have a relatively good prognosis. Therefore, this further indirectly supports that *TIMELESS* is a carcinogenic gene in glioma. However, the mechanism by which *TIMELESS* participates in the malignant behavior of glioma requires more work to reveal.

*Cell signaling pathways of TIMELESS in glioma*

We divided the tissue samples into a high expression group and low expression group based on the expression level of *TIMELESS*. Then, GSEA analysis was used to reveal the activation of cancer-related cell signaling pathways caused by high expression of *TIMELESS*. Potential cellular molecular pathways were selected out by the consistent results of three databases enriched together (**Table 1**). *TIMELESS* high expression may participate in tumor development by activating cell cycle, mismatch repair, and DNA replication cell signaling pathways (**Figure 5A-C**).

*Abnormally high expression of TIMELESS protein levels in glioma*

We have verified the abnormally high expression of *TIMELESS* in glioma based on mRNA. However, the level of *TIMELESS* protein was unclear. We downloaded the immunohistochemical film of *TIMELESS* protein expression in glioma tissue and normal brain tissue from The Human Protein Atlas website. Then we divided into three groups according to tumor grade, including a normal group, low-grade group, high-grade group. We found that *TIMELESS* protein expression level was markedly higher in both the low-level group and the high-level group than the normal group (**Figure 6**).

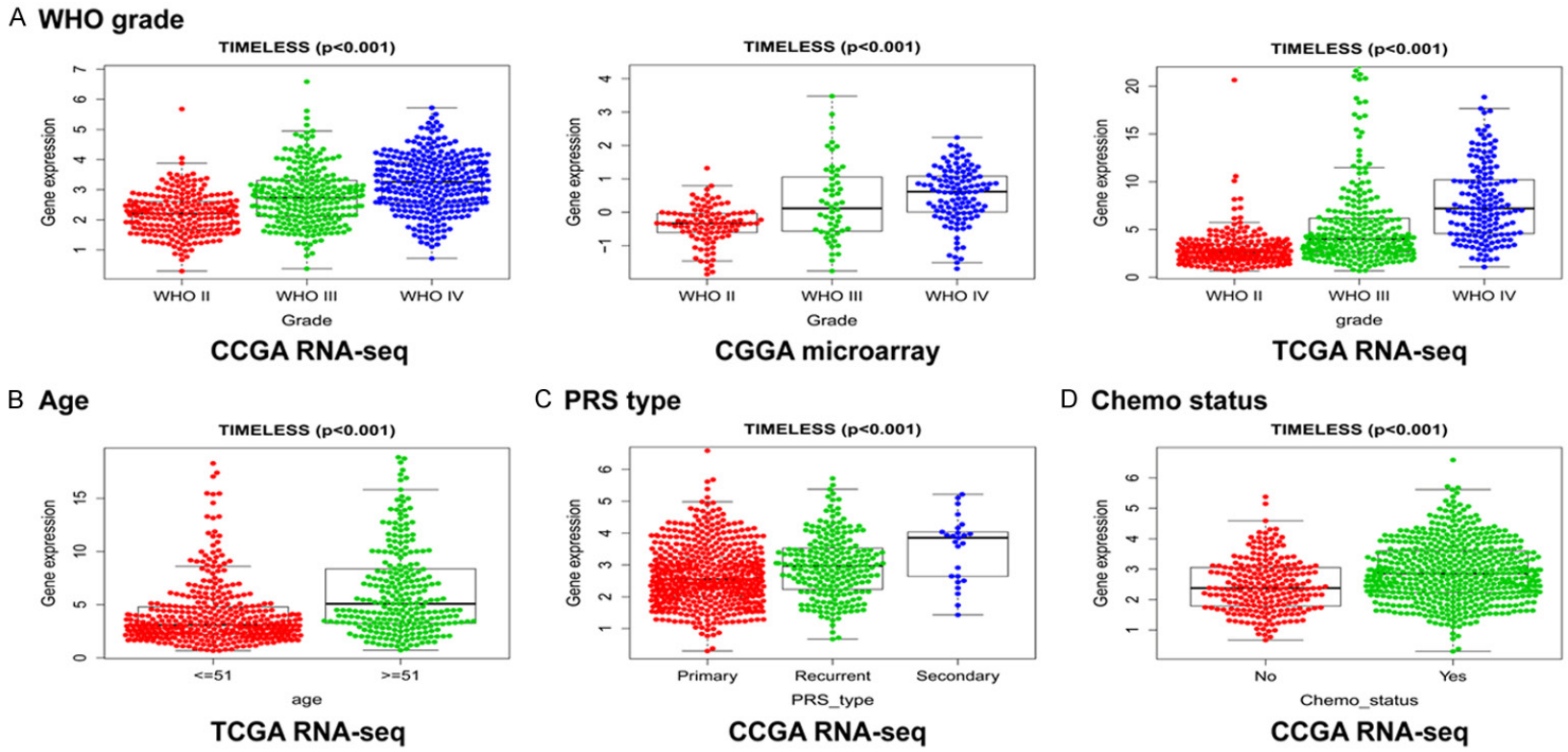
*Drugs that may inhibit TIMELESS*

To find drugs that may inhibit expression of *TIMELESS*, we first performed a correlation analysis between *TIMELESS* and other differentially expressed genes. We selected 10 positively related genes and 10 negatively related genes and drew a circle diagram (**Figure 7**). Next, we uploaded these 20 related genes to the CMap website and screened out 4 small molecule compounds that may inhibit *TIMELESS*, such as 8-azaguanine, gw8510, 6-Thioguanosine, and ursodeoxycholic acid (**Figure 8**; **Table 2**). Some of these drugs have been found to have clear anti-tumor effects in other studies. This supports our predictions.

## Discussion

In recent years, many studies have shown that *TIMELESS* can affect the process of tumor formation through proliferation, migration, and inhibition of apoptosis [19, 27, 28]. However, the effect of *TIMELESS* on the prognosis of glioma patients and its molecular mechanism has not been elucidated. We used the advantage of large samples to reveal the role of *TIMELESS* in glioma. From mRNA to protein levels, we found that *TIMELESS* was highly expressed in glioma relative to normal brain tissue (**Figures 1**, **6**). At the same time, our conclusions were verified by previous studies. For example, Bianco et al. found that *TIMELESS* was abnormally highly expressed in non-small-cell lung cancers and breast cancers [29]. Zhou et al. confirmed *TIMELESS* expression was higher in cervical cancer from both public databases and clinical samples [30]. Wang also verified that *TIMELESS* was highly expressed in glioma [31]. However, the study did not elaborate on the relationship between *TIMELESS* and the clinical characteristics of glioma patients, or the underlying molecular mechanism.

We first found *TIMELESS* was associated with prognosis in glioma patients. As described in **Figures 2**, **3**, high expression of *TIMELESS* was an independent risk factor for the prognosis of glioma patients and had clinical diagnostic value. In other tumors, we also found that


A WHO grade
TIMELESS (p<0.001)
Gene expression
WHO II
WHO III
WHO IV
Grade
CCGA RNA-seq
TIMELESS (p<0.001)
Gene expression
WHO II
WHO III
WHO IV
Grade
CGGA microarray
TIMELESS (p<0.001)
Gene expression
WHO II
WHO III
WHO IV
grade
TCGA RNA-seq
B Age
TIMELESS (p<0.001)
Gene expression
<=51
>=51
age
TCGA RNA-seq
C PRS type
TIMELESS (p<0.001)
Gene expression
Primary
Recurrent
Secondary
PRS_type
CCGA RNA-seq
D Chemo status
TIMELESS (p<0.001)
Gene expression
No
Yes
Chemo_status
CCGA RNA-seq

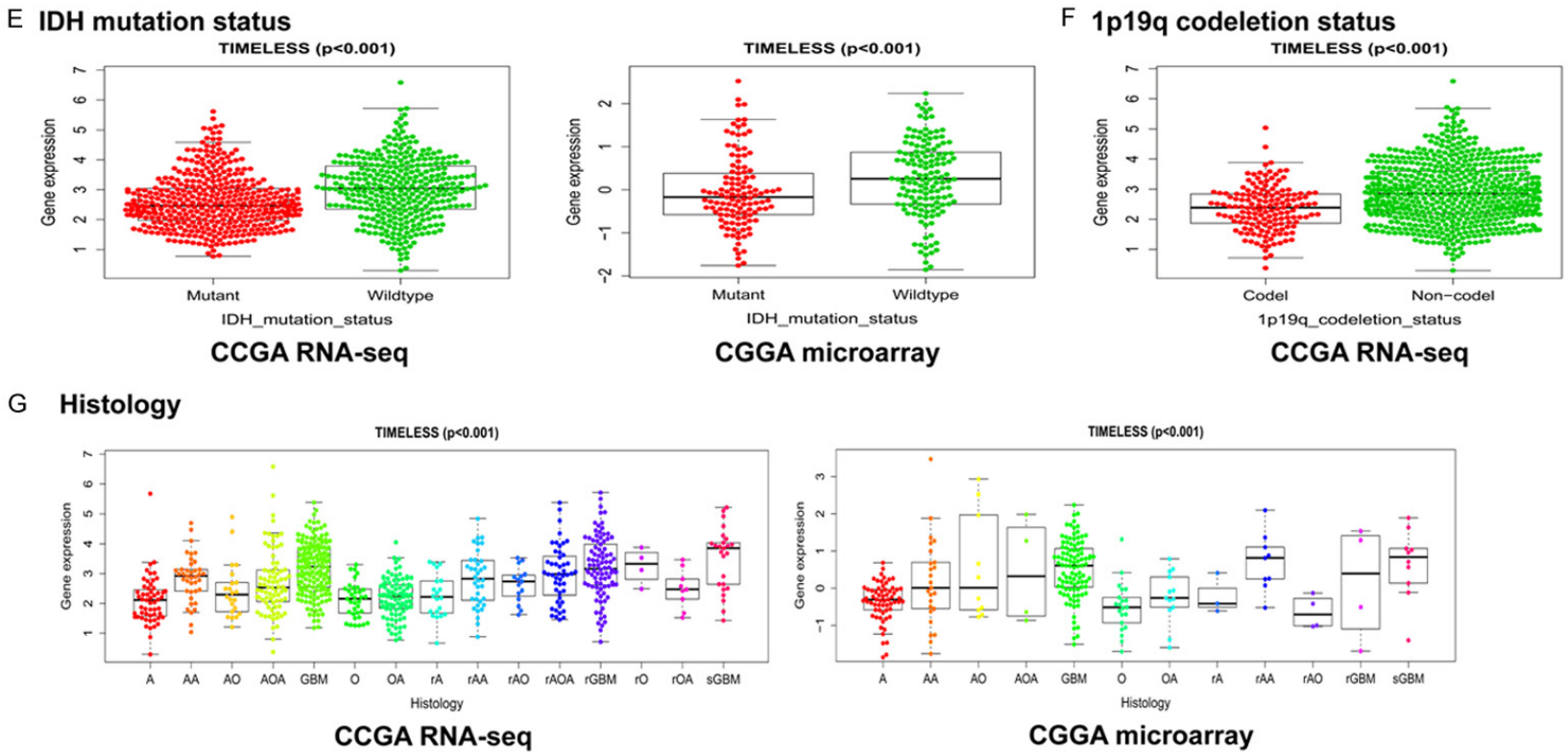



**Figure 4.** Correlation analysis of TIMELESS and clinical features in CGGA RNA-seq, CGGA microarray, and TCGA RNA-seq datasets. A. WHO grade. B. Age. C. PRS type. D. Chemo status. E. IDH mutation status. F. 1p19q codeletion status. G. Histology.

**Table 1.** Gene set enriches high *TIMELESS* in three databases

| Gene set name | CGGA RNA-seq | | CGGA microarray | | TCGA RNA-seq | |
|---|---|---|---|---|---|---|
| | NOM *P*-value | FDR q-value | NOM *P*-value | FDR q-value | NOM *P*-value | FDR q-value |
| Cell cycle | 0.000 | 0.061 | 0.000 | 0.000 | 0.000 | 7.52E-04 |
| DNA replication | 0.000 | 0.108 | 0.000 | 0.018 | 0.000 | 0.005 |
| Mismatch repair | 0.002 | 0.113 | 0.008 | 0.043 | 0.000 | 0.005 |
| Base excision | 0.002 | 0.203 | 0.039 | 0.125 | 0.000 | 0.005 |

Gene sets with NOM *P*-value <0.05 and FDR q-value <0.25 were considered significantly enriched, NOM: nominal; FDR: false discovery rate.

*TIMELESS* was closely related to clinical prognosis. For instance, *TIMELESS* in lung cancer promotes the malignant characteristics of tumors and leads to a poor prognosis for patients [32]. Zhang et al. found that *TIMELESS* promotes the malignant progression of tumors and poor prognosis in cervical carcinoma [33]. JuAs in **Figure 4**, it indicates that the expression level of *TIMELESS* was positively correlated with the age of patients and the grade of glioma. As we all know, grades III-IV glioma show diffuse infiltrating growth with high heterogeneity. The tumor resists postoperative radiotherapy and chemotherapy, and prognosis is less than ideal [34]. This is completely consistent with findings. Therefore, we speculate that the high expression of *TIMELESS* is mainly involved in the malignant behavior at a late stage of the tumor, which provides a direction for the next step to explore the specific molecular mechanism of *TIMELESS*.

We used GSEA to analyze the three databases and found that the high expression of *TIMELESS* may participate in the malignant progression of the tumor through the cell cycle, mismatch repair, and DNA replication cell signaling pathways. GSEA and enrichment analysis are two commonly used to predict the molecular mechanism of participation. Enrichment analysis often focuses on a part of significantly different gene groups, which makes it easy to miss some genes that are not significantly differentially expressed but have important biologic significance [33]. GSEA does not need to set the threshold of differential genes in advance and analyze several genes from the expression profile level according to the overall trend of actual data [35]. In addition, our predicted pathway has been reported to be associated with tumorigenicity. Emmanuel et al. found that mismatch repair genes mutations in ovarian cancer can promote the growth of tumor cells to a distance and worsen the clinical physiology of patients [36]. Kristin et al. discovered that the excision repair system was abnormally high in the early stage of many tumor cells and promotes tumor malignant progression [37]. Evan et al. found that the process of tumor formation was diverse, but the cell cycle played a role that cannot be ignored [38]. Therefore, through the above description, we can conclude that the abnormal increase of *TIMELESS* in glioma may lead to poor prognosis through a variety of cell signaling pathways.

Using the advantage of public databases, we have demonstrated that the high expression of *TIMELESS* was closely related to the malignant behavior of high-grade glioma. However, there are some flaws in this experiment. First, the clinical information of some samples in the database was incomplete, the treatment plan was not clear, and the database also was regional. We used a combination of multiple databases to expand the sample size to make up for this problem and avoid sample bias. Second, the cell signaling pathway of GSEA analysis was not proven with traditional experiments. However, the GSEA method has been reported in many studies and has certain feasibility. *TIMELESS* may have other effects and this article cannot specifically reveal all their pathogenesis.

In conclusion, in this study, we found that the high expression of *TIMELESS* was closed related to the prognosis of glioma patients and was an independent prognostic factor. We believe that *TIMELESS* can also be a molecular marker for screening for glioma and may be a target for gene therapy.

**Acknowledgements**

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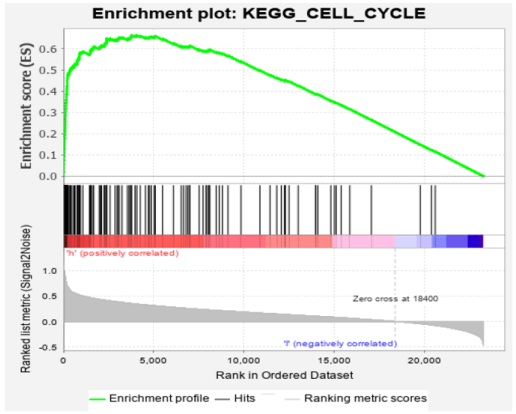
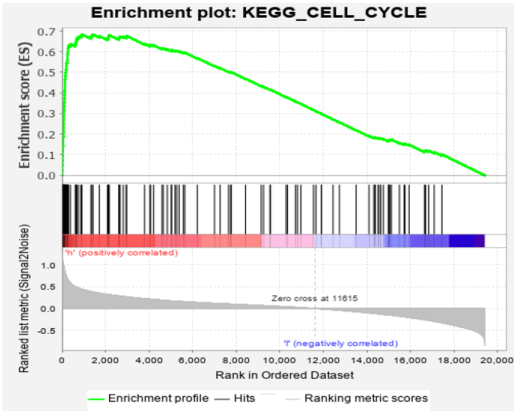
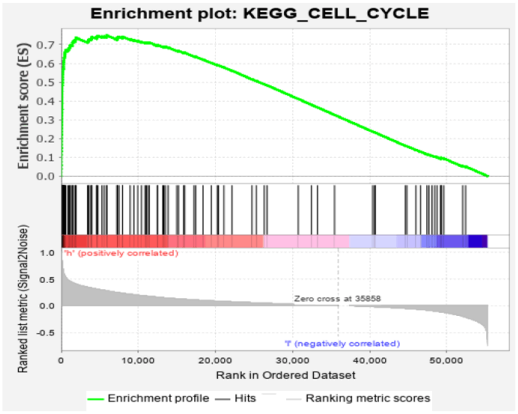
A




CGGA RNA-seq

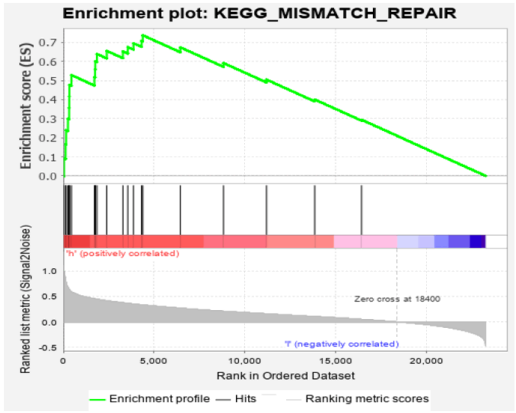



CGGA microarray

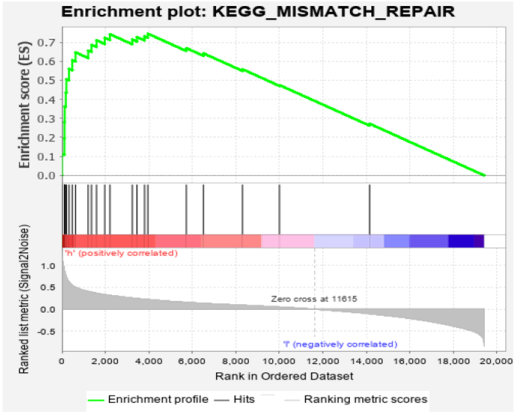



TCGA RNA-seq

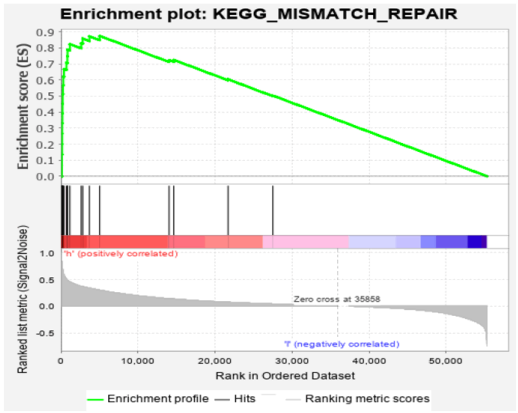
B




CGGA RNA-seq




CGGA microarray




TCGA RNA-seq

C

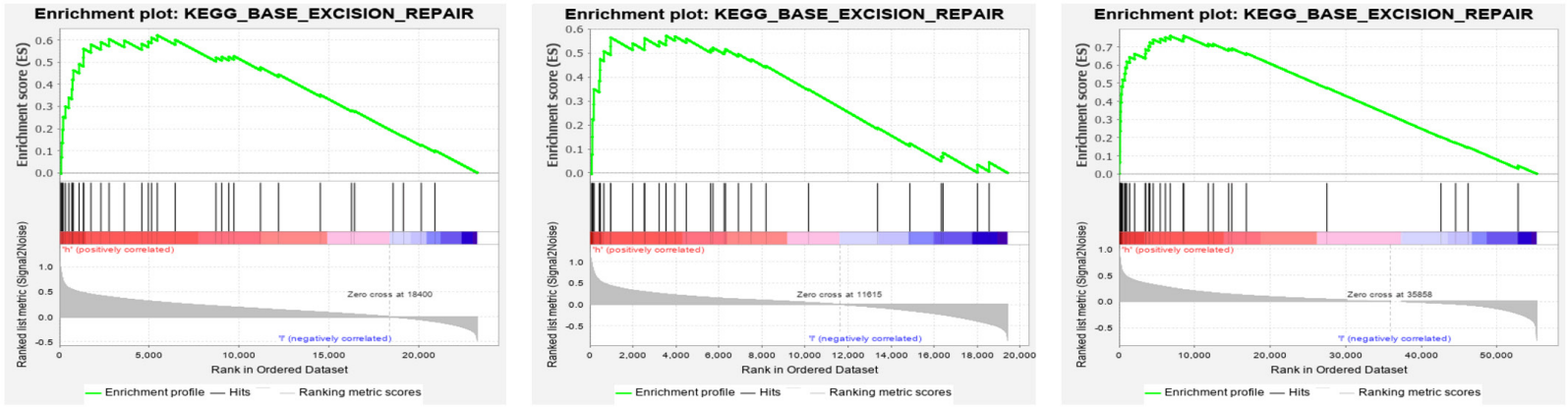



**Figure 5.** Enrichment results of GSEA based on different datasets. A. Cell-cycle. B. Mismatch-repair. C. Base excision repair. GSEA: gene set enrichment analysis.

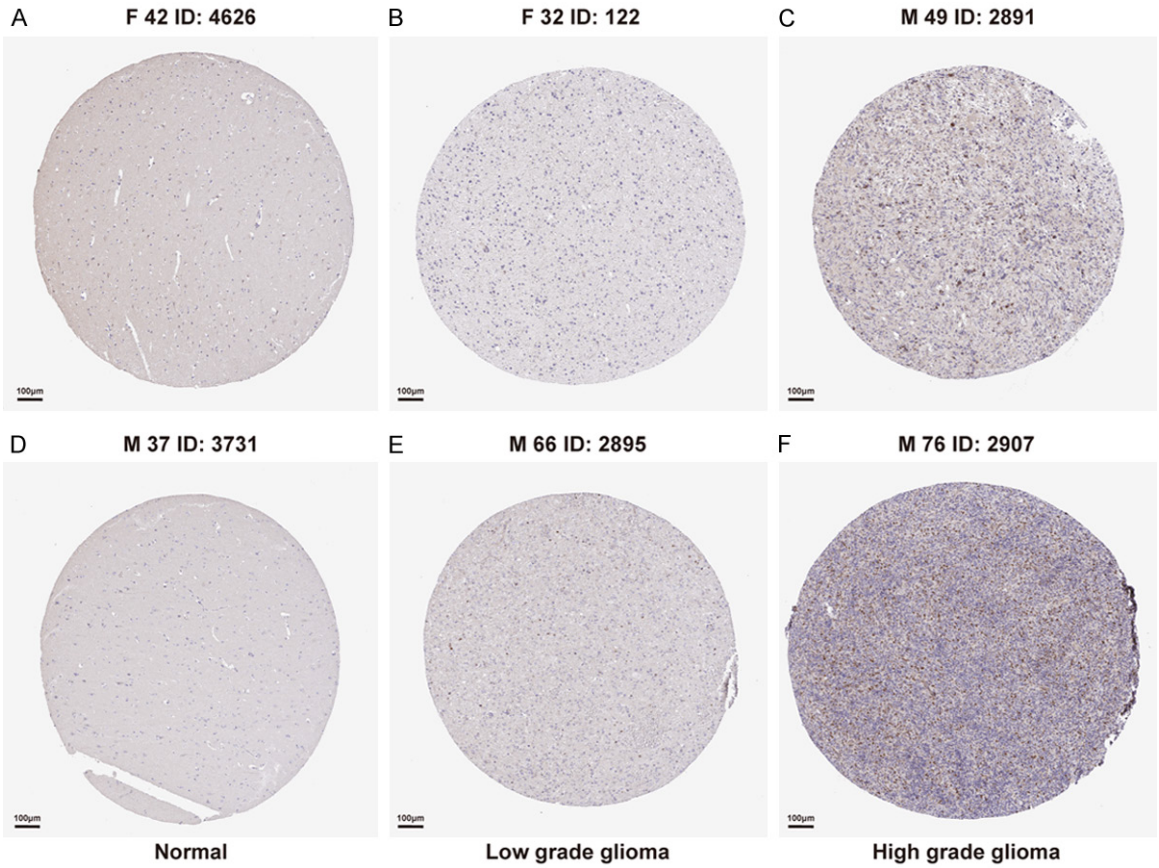
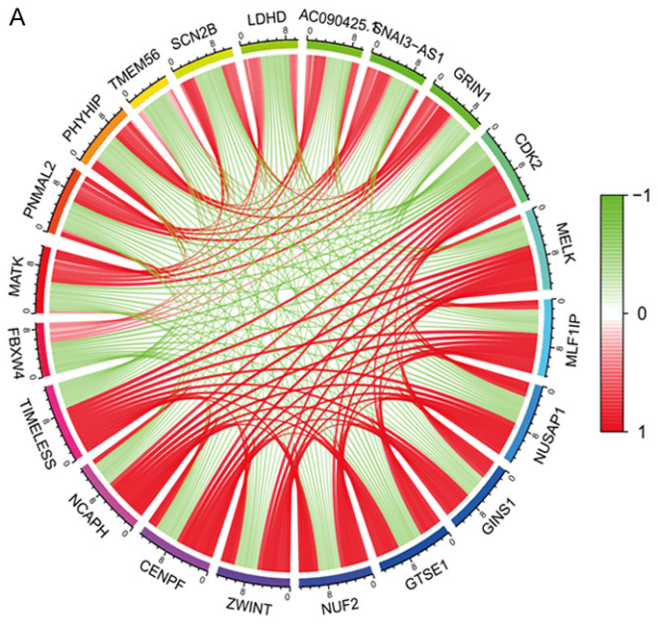



**Figure 6.** Expression of TIMELESS protein from glioma tissue and normal brain tissue. A, B. In normal brain tissue. C, D. In low grade glioma tissue. E, F. In high grade glioma tissue. M: male, F: female. ID from ATLAS.




B

| No. | Gene | Correlation index | *P*-value |
|---|---|---|---|
| 1 | *NCAPH* | 0.881 | 0 |
| 2 | *CENPF* | 0.878 | 0 |
| 3 | *ZWINT* | 0.875 | 1.19E-322 |
| 4 | *NUF2* | 0.874 | 1.88E-320 |
| 5 | *GINS1* | 0.869 | 3.13E-313 |
| 6 | *GTSE1* | 0.869 | 1.81E-313 |
| 7 | *MLF1IP* | 0.866 | 3.97E-308 |
| 8 | *NUSAP1* | 0.866 | 1.56E-307 |
| 9 | *CDK2* | 0.865 | 1.88E-306 |
| 10 | *MELK* | 0.865 | 1.40E-306 |
| 11 | *GRIN1* | -0.441 | 9.96E-50 |
| 12 | *SNAI3-AS1* | -0.446 | 5.69E-51 |
| 13 | *AC090425.1* | -0.454 | 6.82E-53 |
| 14 | *LDHD* | -0.459 | 3.66E-54 |
| 15 | *SCN2B* | -0.46 | 1.48E-54 |
| 16 | *TMEM56* | -0.468 | 1.38E-56 |
| 17 | *PHYHIP* | -0.477 | 4.61E-59 |
| 18 | *MATK* | -0.486 | 2.49E-61 |
| 19 | *PNMAL2* | -0.486 | 1.88E-61 |
| 20 | *FBXW4* | -0.487 | 8.17E-62 |

**Figure 7.** Correlation analysis between TIMELESS and other differential genes. A, B. Screened out the top 10 differential genes with positive and negative correlations.

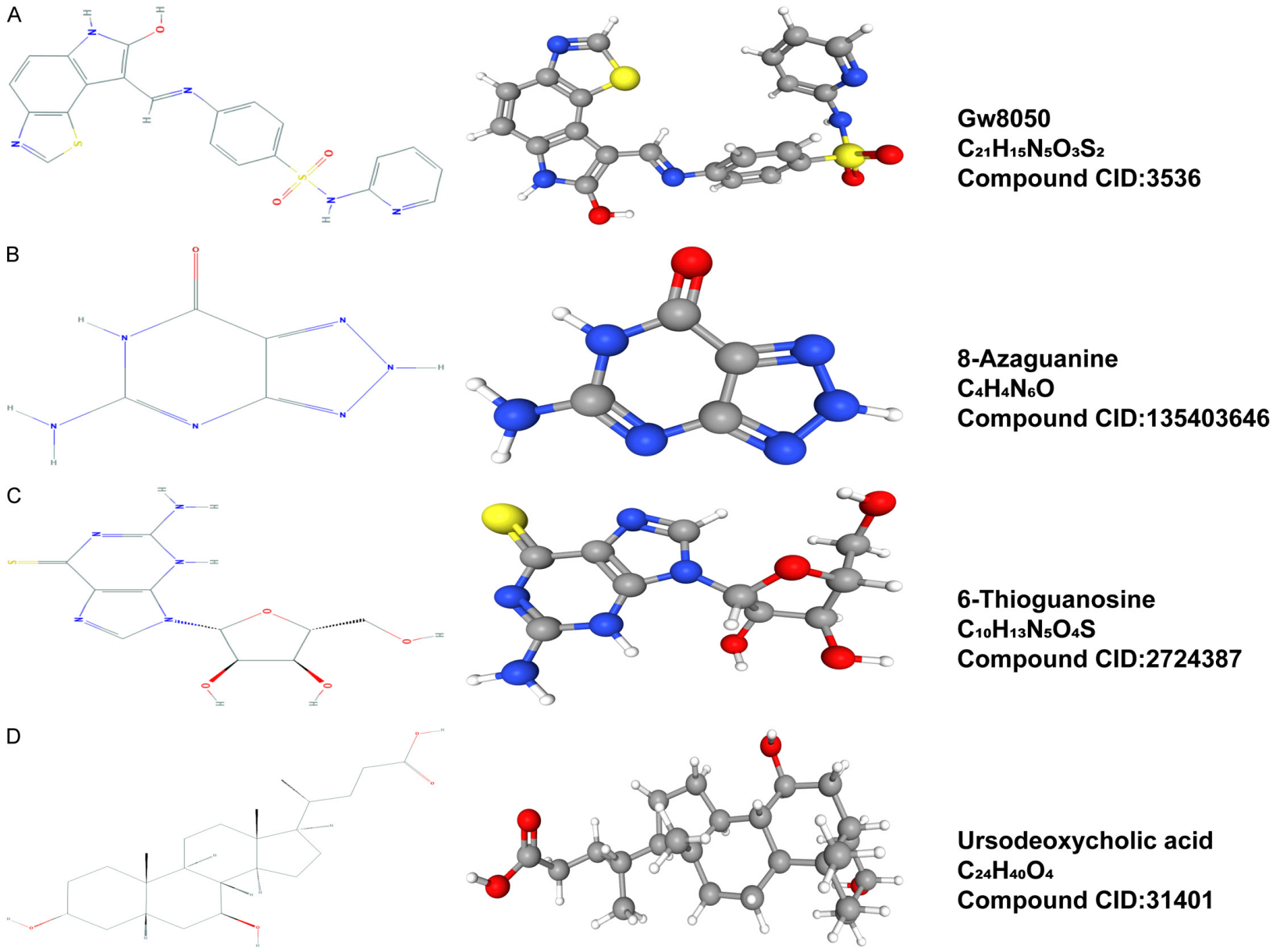



**Figure 8.** Small molecule compounds may inhibit TIMELESS expression. A. Gw8510. B. 8-azaguanine. C. 6-thioguanosine. D. Ursodeoxycholic acid.

**Table 2.** Small molecule compounds predicted by CMap

| No. | Name | Enrichment | *P* |
|---|---|---|---|
| 1 | GW-8510 | -0.929 | 0.00002 |
| 2 | Ursodeoxycholic acid | -0.885 | 0.00300 |
| 3 | 6-thioguanosine | -0.882 | 0.00048 |
| 4 | 8-azaguanine | -0.786 | 0.00426 |

Enrichment <-0.7, *P*<0.05.

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## Disclosure of conflict of interest

None.

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**Table S1.** Characteristics of patients with glioma based on CGGA RNA-seq data

| Characteristics | | Number of cases | Percentages (%) |
|---|---|---|---|
| Gender | Male | 306 | 40.91 |
| | Female | 442 | 59.09 |
| Age | ≤41 | 341 | 45.59 |
| | >41 | 407 | 54.41 |
| Grade | WHO II | 218 | 29.14 |
| | WHO III | 240 | 32.09 |
| | WHO IV | 290 | 38.77 |
| PRS type | Primary | 501 | 66.98 |
| | Recurrent | 222 | 29.68 |
| | Secondary | 25 | 3.34 |
| Radio status | Yes | 625 | 83.56 |
| | No | 123 | 16.44 |
| Chemo status | Yes | 520 | 69.52 |
| | No | 228 | 30.48 |
| Histology | Astrocytoma | 55 | 7.35 |
| | Anaplastic astrocytoma | 39 | 5.21 |
| | Anaplastic oligodendroglioma | 22 | 2.94 |
| | Anaplastic oligoastrocytoma | 80 | 10.70 |
| | Glioblastoma | 175 | 23.40 |
| | Oligodendroglioma | 35 | 4.68 |
| | Oligoastrocytoma | 95 | 12.70 |
| | Relapse astrocytoma | 20 | 2.67 |
| | Relapse anaplastic astrocytoma | 36 | 4.81 |
| | Relapse anaplastic oligodendroglioma | 15 | 2.01 |
| | Relapse anaplastic oligoastrocytoma | 48 | 6.42 |
| | Relapse glioblastoma | 90 | 12.03 |
| | Relapse oligodendroglioma | 4 | 0.53 |
| | Relapse oligoastrocytoma | 9 | 1.20 |
| | Secondary relapse glioblastoma | 25 | 3.34 |
| IDH mutation status | Mutant | 409 | 54.68 |
| | Wildtype | 339 | 45.32 |
| 1p19q codeletion status | Codeletion | 155 | 20.72 |
| | Non-codeletion | 593 | 79.28 |

**Table S2.** Characteristics of patients with glioma based on CGGA microarray data

| Characteristics | | Number of cases | Percentages (%) |
|---|---|---|---|
| Gender | Male | 153 | 57.09 |
| | Female | 115 | 42.91 |
| Age | ≤42 | 138 | 51.49 |
| | >42 | 130 | 48.51 |
| Grade | WHO II | 100 | 37.31 |
| | WHO III | 52 | 19.40 |
| | WHO IV | 116 | 43.28 |
| PRS type | Primary | 238 | 88.81 |
| | Recurrent | 20 | 7.46 |
| | Secondary | 10 | 3.73 |
| Radio status | Yes | 240 | 89.55 |
| | No | 28 | 10.45 |
| Chemo status | Yes | 145 | 54.10 |
| | No | 123 | 45.90 |
| Histology | Astrocytoma | 63 | 23.51 |
| | Anaplastic astrocytoma | 25 | 9.33 |
| | Anaplastic oligodendroglioma | 10 | 3.73 |
| | Anaplastic oligoastrocytoma | 4 | 1.49 |
| | Glioblastoma | 102 | 38.06 |
| | Oligodendroglioma | 21 | 7.84 |
| | Oligoastrocytoma | 13 | 4.85 |
| | Relapse astrocytoma | 3 | 1.12 |
| | Relapse anaplastic astrocytoma | 9 | 3.36 |
| | Relapse anaplastic oligodendroglioma | 4 | 1.49 |
| | Relapse glioblastoma | 4 | 1.49 |
| | Secondary relapse glioblastoma | 10 | 3.73 |
| IDH mutation status | Mutant | 121 | 45.15 |
| | Wildtype | 147 | 54.85 |

**Table S3.** Characteristics of patients with glioma based on TCGA RNA-seq data

| Characteristics | | Number of cases | Percentages (%) |
|---|---|---|---|
| Gender | Male | 377 | 57.73 |
| | Female | 276 | 42.27 |
| Age | ≤51 | 394 | 60.34 |
| | >51 | 259 | 39.66 |
| Grade | WHO II | 238 | 36.45 |
| | WHO III | 256 | 39.20 |
| | WHO IV | 159 | 24.35 |