Hypomethylation-induced prognostic marker zinc finger DHHC-type palmitoyltransferase 12 contributes to glioblastoma progression

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Background: Glioma is the most common intracranial primary malignancy, characterized by abnormal signal transductions caused by transcriptional and post-transcriptional regulators. Studies show the palmitoylation of oncoproteins and tumor suppressors participate in cancer progression, while studies of protein S-palmitoyltransferases in glioma are limited. A systematic analysis of zinc finger DHHC-type palmitoyltransferases (ZDHHC) in glioma is still lacking.

Methods: A prognostic heatmap and Kaplan-Meier overall survival plot of 24 members of the ZDHHC family in pan-cancer created. The expression and prognostic significance of ZDHHC12 was analyzed by using Gene Expression Profiling Interactive Analysis (GEPIA) and PrognoScan. DBTRG and U251 cells with silenced ZDHHC12 expression were constructed and used for cell counting kit-8 (CCK-8), Transwell assay and wound healing assay *in vitro*.

Results: Here, we first conducted expression and prognostic analyses of 24 ZDHHCs from The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA), and other glioma datasets. We found ZDHHC12 to be the only unfavorable prognostic marker in glioma. The function of ZDHHC12 in glioma was then investigated with loss-of-function strategies and in vitro cell assays. Results showed that ZDHHC12 knockdown remarkably reduced the growth, migration, and invasion capabilities in DBTRG and U251 cell lines, suggesting that ZDHHC12 may contribute to malignant behavior in glioma cells. Finally, the molecular basis for ZDHHC12 expression in glioma was analyzed, and DNA hypomethylation was found to be responsible for increased ZDHHC12 mRNA expression and related prognoses.

Conclusions: ZDHHC12 positively promoted the proliferation and migration of glioma cells. Decreased DNA methylation may lead to increased ZDHHC12 expression in gliomas. This study may deepen the understanding of glioma progression and therapeutics.

Keywords: Glioma; ZDHHC12; prognosis; hypomethylation

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Introduction

Gliomas are the most common intracranial primary malignancy, with glioblastoma (GBM) the most malignant form (1). Based on their histological properties, gliomas have been categorized into the World Health Organization (WHO) classifications of astrocytic, oligodendroglial, or ependymal tumors, which indicate different degrees of malignancy (2). Accumulating evidence shows that gliomas are genetic, epigenetic, and highly heterogeneous (3). Glioma development, such as apoptosis and immune evasion, is characterized by active and passive DNA demethylation processes, that promote certain apoptosis and epigenetic reprogramming of immune system participants (4). The abnormal signal transductions caused by transcriptional and post-transcriptional regulators underlie glioma pathogenesis, progression, and therapeutic responses (5,6). Although diagnostic (7,8) and therapeutic advances (e.g., surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy) (1,5,9) have been achieved, outcomes in patients with glioma still remain unsatisfactory. Therefore, it is necessary to identify novel genes and prognostic markers in glioma.

Protein S-palmitoylation is a reversible post-translational modification and one of the most common lipid modifications (10). Protein S-palmitoylation has been reported to play a role in protein stability, transport, and human disease processes (11,12). Protein palmitoylation is regulated by palmitoyl S-acyltransferases from the DHHC family, while the removal of palmitate is catalyzed by serine hydrolases, including acyl-protein thioesterases (APTs) (13). Studies have shown that the palmitoylation of oncoproteins and tumor suppressors participate in cancer progression (14-17). However, studies of protein palmitoylation in glioma are limited. In p53-mutated glioma, Chen *et al*. proved that zinc finger DHHC-type palmitoyltransferase 5 (*ZDHHC5*) acts as an oncogene via *EZH2* palmitoylation (18). Zhang *et al*. recently found that *DHHC9*-mediated *GLUT1* S-palmitoylation promotes glioblastoma glycolysis and tumorigenesis (19). In a study by Fan *et al.*, *ZDHHC15*-mediated *GP130* palmitoylation was found to be involved in the effect of local anesthetics on the repression of growth and self-renewal of glioblastoma stem cells (20). In another study, Fan *et al.* found that Smad3 palmitoylation by *ZDHHC19* promoted the activation of transforming growth factor-beta (*TGF*β) in the mesenchymal subtype of GBM (21). Differential expression, prognostic analysis and functional correlation analysis of 10 kinds of ZDHHC in renal clear cell carcinoma were systematically investigated (22). However, there is still a lack of systematic analyses of *ZDHHCs* in glioma.

ZDHHC12 is one of the protein palmitoylation regulated by palmitoyl s-acyltransferases from the DHHC family (12). Currently, there are few studies on the occurrence and progression of tumors related to ZDHHC12, which is why we chose ZDHHC12 as the main research object. This study analyzed the expression and prognostic of *ZDHHCs* *in* glioma and found *ZDHHC12* to be the hub gene. The function of ZDHHC12 in glioma was then investigated using loss-of-function strategies and *in vitro* cell assays. Finally, the molecular basis for *ZDHHC12* expression in glioma was analyzed, and DNA hypomethylation was found to be responsible for increased *ZDHHC12* mRNA expression and related prognoses. Our research aimed to reveal the clinical significance and cellular function of *ZDHHC12* in glioma were revealed for the first time, which could provide a theoretical basis for ZDHHC12 as a marker for early screening of glioma in the future, and might deepen the understanding of glioma progression and therapeutics. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-520/rc).

Methods

Expression and prognosis analyses

A prognostic heatmap and Kaplan-Meier overall survival plot of 24 members of the *ZDHHC* family in pan-cancer were created, and the expression of *ZDHHC12* in pan-cancer and glioma was investigated using GEPIA 2.0 (23). The expression and methylation of *ZDHHC12* in the Chinese Glioma Genome Atlas (CGGA) samples (24) were analyzed using the CGGA website: http://cgga.org.cn/. The prognostic significance of *ZDHHC12* was analyzed for other datasets using PrognoScan (25). The GSE4271 dataset (26-28) included 77 primary high-grade astrocytomas and 23 matched recurrences. The GSE4412 dataset included 85 gliomas from 74 patients, and platform were Affymetrix HG-U133A and HG-U133B arrays (29). This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture

DBTRG and U251 glioma cell lines were preserved in our laboratory. Minimum essential medium-alpha (MEM)

was used to culture cell lines with 10% fetal bovine serum (FBS) which is heat-inactivated, penicillin (100 IU/mL) and streptomycin (100 µg/mL) were used to prevent bacterial infection. Cells were cultured at 37 ℃ in humidified incubators, with 95% humidity and 5% carbon dioxide.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from the cells using a TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and then determined its concentration and purity. The total RNA was reverse transcribed into cDNA using a ReverTra Ace qPCR RT kit (Toyobo Life Science) following the manufacturer's instructions. Primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Two-step quantitative polymerase chain reaction (qPCR) was performed, also using the ReverTra Ace qPCR RT kit (Toyobo Life Science), with *GAPDH* serving as the internal reference. The primers were used as following:

(F) 5'-GTGCTGACCTGGGGAATCAC-3' and (R) 5'-CTGCACATTCACGTAGCCA-3' for *ZDHHC12*; and (F) 5'-GGAGCGAGATCCCTCCAAAAT-3' and (R) 5'-GGCTGTTGTCATACTTCTCATGG-3' for *GADPH*.

Short hairpin RNAs targeting ZDHHC12 and cell transfection

To silence the expression of *ZDHHC12* in glioma cells, 3 short hairpin (sh) RNAs were designed and synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). The shRNA sequences were: shRNA-1: 5'-GGAATCACGCTGGTGCTCTTC-3'; shRNA-2: 5'-GAATCACGCTGGTGCTCTTCC-3' and shRNA-3: 5'- GCACGATACCGAGCTGCGGCA-3'. The shRNAs were cloned into PcDNA 3.1 and prepared for subsequent transfection. DBTRG and U251 cells were used for transfection at the exponential stage. 1×10^6 cells were cultured in 6-well plates before transfection (with 2 mL above-mentioned medium for 24 hours) until they were 90% confluent.

Cell counting kit-8 assay (CCK-8)

A CCK-8 assay was conducted as per our previous study (30). Briefly, cellular viability was determined using a CCK-8 (Beyotime Biotechnology). After transfection, DBTRG and U251 cells were plated into 96-well plates (at a density of 500 cells per well). Then, incubated 10 µL CCK-8 solution with the cell medium to each well for another 2 hours at 37 ℃ daily. Using Multiskan FC Microplate spectrophotometer to detect the absorbance of each well at 570 nm. Data were showed as mean ± standard deviation (SD) and comparisons were analyzed by 2-way repeated measure analysis of variance (RM ANOVA) using GraphPad Prism 8.0.

Wound-healing assay

A wound-healing assay was conducted as per our previous study (30). Briefly, DBTRG and U251 cells were seeded in a 6-well plate evenly with the cell concentration of 1×10^6 per well. To inhibit cell division, serum-free medium containing 1 mM mitomycin was used to replace the complete medium. When the cells covered 90% of the plate base, the plate was perpendicularly scratched with a 10 µL sterile pipette tip Then removed the culture medium in the plate and softly washed 3 times with phosphate-buffered saline (PBS). To make the visual field clear for photography phase, the cell debris was carefully rinsed off. A medium containing 1% fetal bovine serum (FBS) was added to the 6-well plate. Introduced 3 mm wound of each plate across the diameter.

Transwell migration assay

A transwell migration assay was conducted as per our previous study (30). Briefly, cells in the logarithmic growth phase were seeded in the upper transwell chamber insert (Corning, USA) at a density of 2×10^4 cells per well. To inhibit cell division, serum-free medium containing 1 mM mitomycin was used to replace the complete medium. A 24-well plate was used to place the chamber, where the upper chamber containing FBS-free medium and the lower chamber containing 10% FBS complete medium. Cells cultured for 24 hours. Then discarded the medium and stained the cells with 1% crystal violet solution in order to present the amount of migrated cells.

Statistical analysis

Comparisons were calculated using 2-sided, unpaired *t*-tests or 2-way ANOVAs in GraphPad Prism 8.0. and data were presented as means ± SDs. All assays were replicated 3 times. A P value of less than 0.05 was considered statistically significant.

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Results

ZDHHC12 expression increased and might predict poor prognosis in glioma

A pan-cancer prognosis heatmap was constructed using GEPIA 2.0 to investigate the prognostic significance of 24 members of the *ZDHHC* family in multiple cancer types included in The Cancer Genome Atlas (TCGA) database (23). Only *ZDHHC12* showed significant performance as an unfavorable marker in glioblastoma (GBM) (*Figure 1A*). In low grade glioma (LGG), all 13 genes showed significant performance, with 9 genes (*ZDHHC24*, *ZDHHC1*, *ZDHHC18*, *ZDHHC23*, *ZDHHC13*, *ZDHHC7*, *ZDHHC12*, *ZDHHC2*, and *ZDHHC5*) showing unfavorable outcomes (hazard ratio >1, and P<0.05) and 4 genes (*ZDHHC11B*, *ZDHHC16*, *ZDHHC21*, and *ZDHHC22*) showing favorable outcomes (hazard ratio <1, and P<0.05). We chose *ZDHHC12* for subsequent analysis, and its expression was compared in cancer and corresponding normal group (TCGA normal and GTEx data). Of the 33 cancer types analyzed, higher expression of *ZDHHC12* was consistently observed in 21, including LGG and GBM, suggesting that *ZDHHC12* may play an important role in multiple cancer types (*Figure 1B*). The differential expressions of *ZDHHC12* in GBM and LGG can be seen in *Figure 1C*, while *Figure 1D* shows the Kaplan-Meier overall survival plots of *ZDHHC12* in GBM and LGG.

Validating the expression and prognostic relevance of ZDHHC12 in other glioma datasets

To further explore the clinical relevance of *ZDHHC12* in glioma, we used data from the CGGA (24) and other datasets. In 3 of the CGGA datasets (array 325, array 301, and array 693), *ZDHHC12* was found to be clinically relevant for histology types, WHO grades, isocitrate dehydrogenase (IDH) mutation, and 1p/19q co-deletion status. Higher expression was observed in GBM samples than in oligodendroglioma, astrocytoma, and other histological types of glioma (*Figure 2A*). *ZDHHC12* showed consistent grade-dependent expression in groups classified according to the WHO grades (*Figure 2B*). For molecular subtypes, increased expression of *ZDHHC12* was observed in IDH wild-type (IDH-WT) (*Figure 2C*) and non-1p/19q co-deletion (*Figure 2D*) glioma tissue. Finally, the Kaplan-

Meier overall survival plots for the 3 datasets also supported TCGA datasets as unfavorable significant prognostic markers (P<0.0001) (*Figure 2E*).

The prognostic significance of *ZDHHC12* was then analyzed in other datasets using PrognoScan (25). *ZDHHC12* showed prognostic significance in 2 datasets: GSE4271 (*Figure 3A*) and GSE4412 (*Figure 3B*). The GSE4271 dataset comprised high-grade astrocytoma samples, and GSE4412 included diffuse infiltrating glioma samples. Together, these results suggest that *ZDHHC12* is upregulated, correlates with cancer progression, and can predict poor survival outcomes in glioma.

Silencing ZDHHC12 repressed cancer growth, migration, and invasion in glioma

To further explore the function of *ZDHHC12* in glioma, we designed 3 shRNAs to intervene in *ZDHHC12* expression in DBTRG and U251 glioma cells. First, the knockdown efficiency was examined using qRT-PCR, and shRNA2 showed the overall best silencing, with a capability of more than 50% (*Figure 4A*). Therefore, we chose shRNA2 for the following phenotype assays. A CCK8 assay was conducted to study both cell lines with decreased *ZDHHC12* expression. Both cell lines exhibited repressed growth capability in the *ZDHHC12* silenced group at 72 hours (*Figure 4B*), suggesting that *ZDHHC12* may contribute to glioma cell proliferation. Next, the function of *ZDHHC12* on glioma cells was evaluated through transwell and wound healing assays for both cell lines. Compared to cells without *ZDHCC* silencing, *ZDHHC12*-silenced cells had significantly fewer trans-membrane cells at 48 hours in both cell lines (*Figure 4C,4D*, P<0.01). Finally, wound healing assays were conducted to analyze the migration potential of *ZDHHC12* silenced cells. *ZDHHC12* silenced cells showed a wider gap at 48 hours (*Figure 4E,4F*), implying that silencing *ZDHHC12* may also reduce metastasis-related malignant behavior in glioma.

ZDHHC12 hypomethylation to predict prognosis in glioma

Finally, to investigate the molecular basis for increased *ZDHHC12* expression in glioma, we analyzed the methylation levels in the CGGA database samples. Interestingly, in direct contrast to *ZDHHC12* mRNA expression trends, the methylation level of *ZDHHC12*
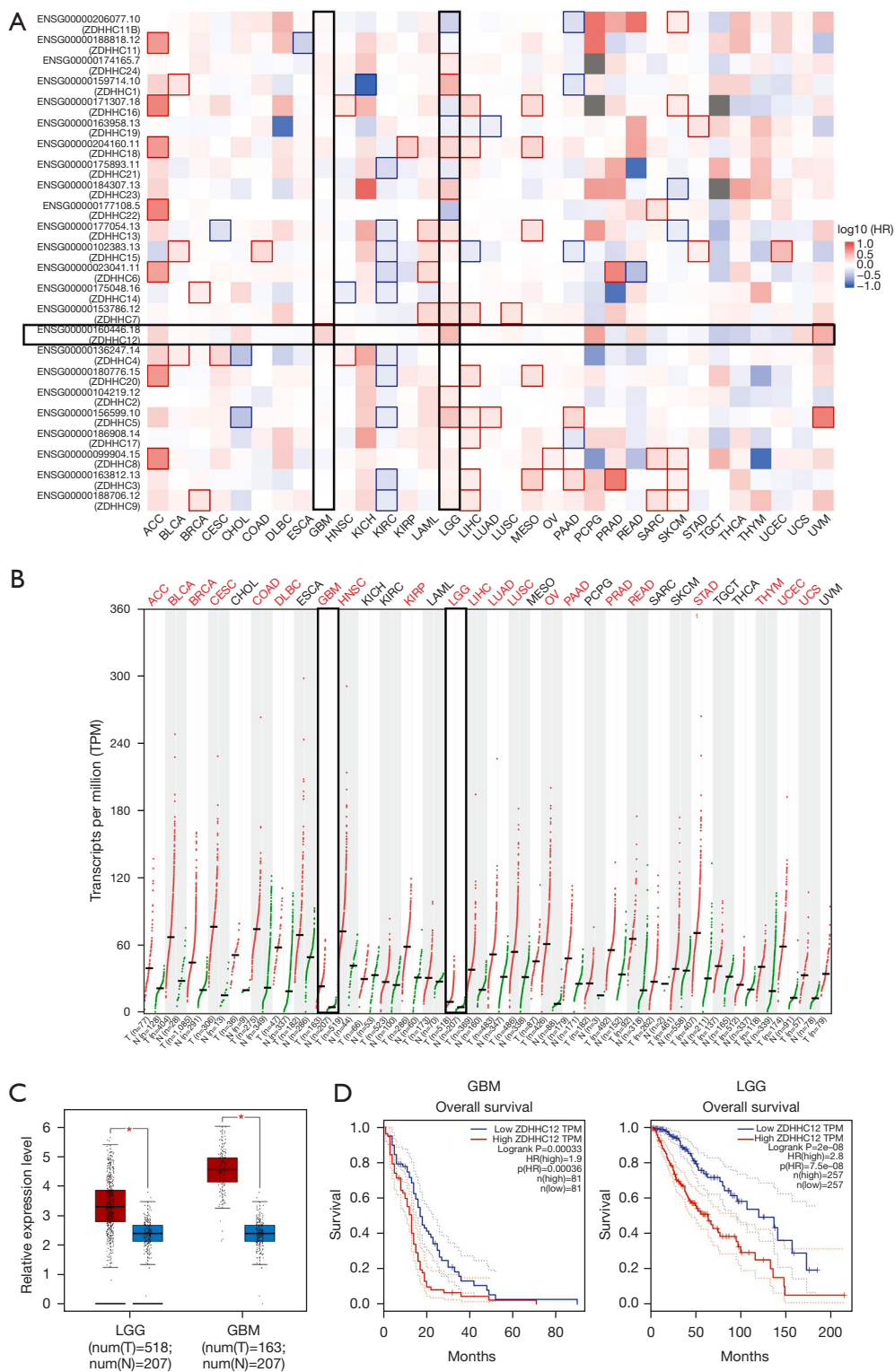
Figure 1 Analysis showing increased expression of ZDHHC12 as a hazardous marker in glioma. (A) Overall survival map of 24 members of the ZDHHC family. (B) Pan-cancer expression analysis showing upregulation of ZDHHC12 in multiple cancer types, including GBM and LGG. (C) Box plot showing significantly increased ZDHHC12 in GBM and LGG datasets. (D) Kaplan-Meier survival plot of ZDHHC12 in GBM and LGG. *, P<0.05. ZDHHC, zinc finger DHHC-type palmitoyltransferase; GBM, glioblastoma multiforme; LGG, low-grade glioma.

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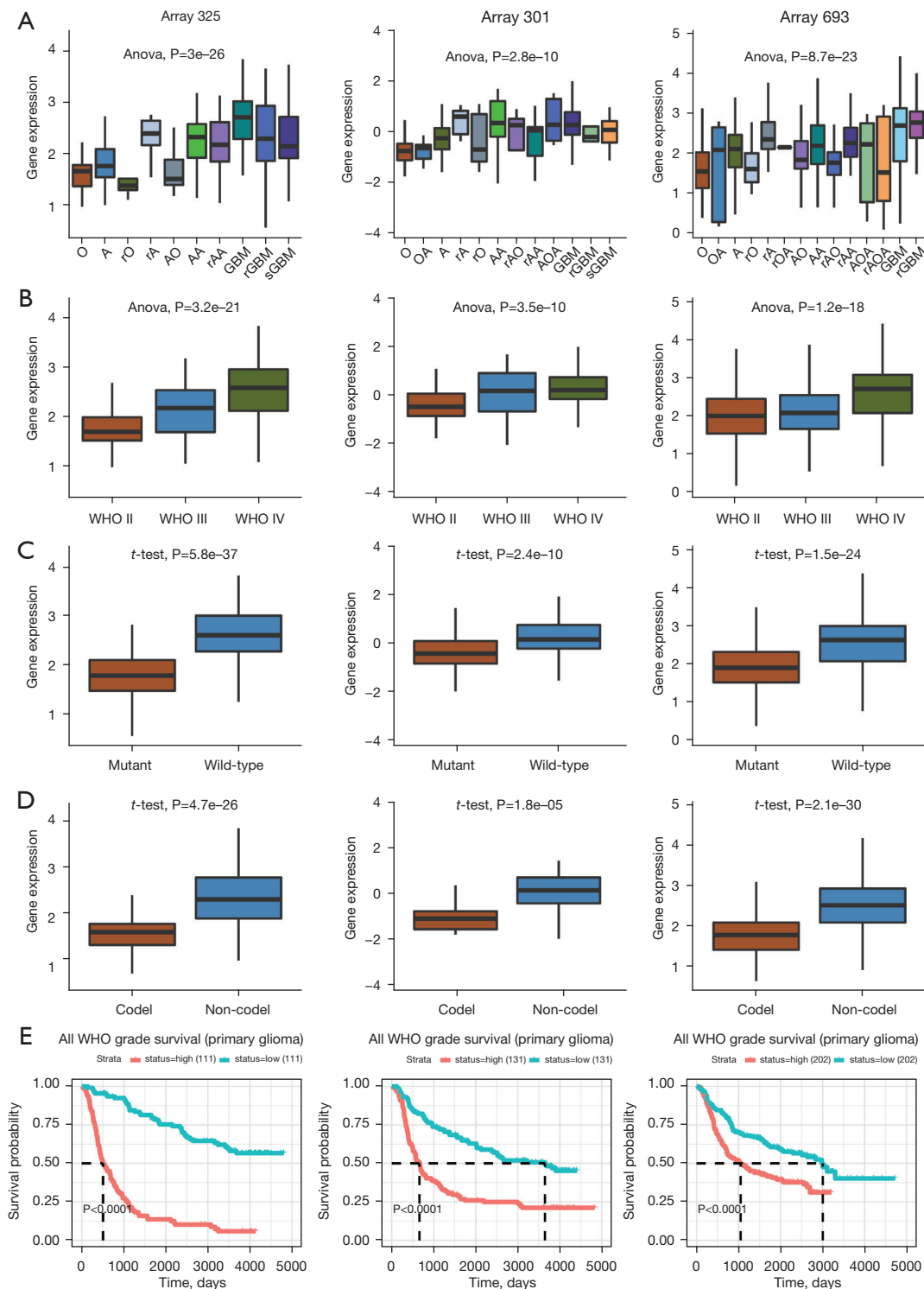
Figure 2 Expression and prognostic performance of ZDHHC12 in CGGA datasets. (A) Expression of ZDHHC12 in different histological types of glioma tissue. (B) Expression of ZDHHC12 in WHO grade II, III, and IV grades glioma tissue. (C) Abundance of ZDHHC12 in IDH mutant and wild-type glioma tissues. (D) ZDHHC12 expression in the 1p/19q co-deletion glioma tissue. (E) Kaplan-Meier survival plot of ZDHHC12 expression in patients with glioma. ZDHHC, zinc finger DHHC-type palmitoyltransferase; CGGA, the Chinese Glioma Gene Atlas; WHO, World Health Organization; IDH, isocitrate dehydrogenase.
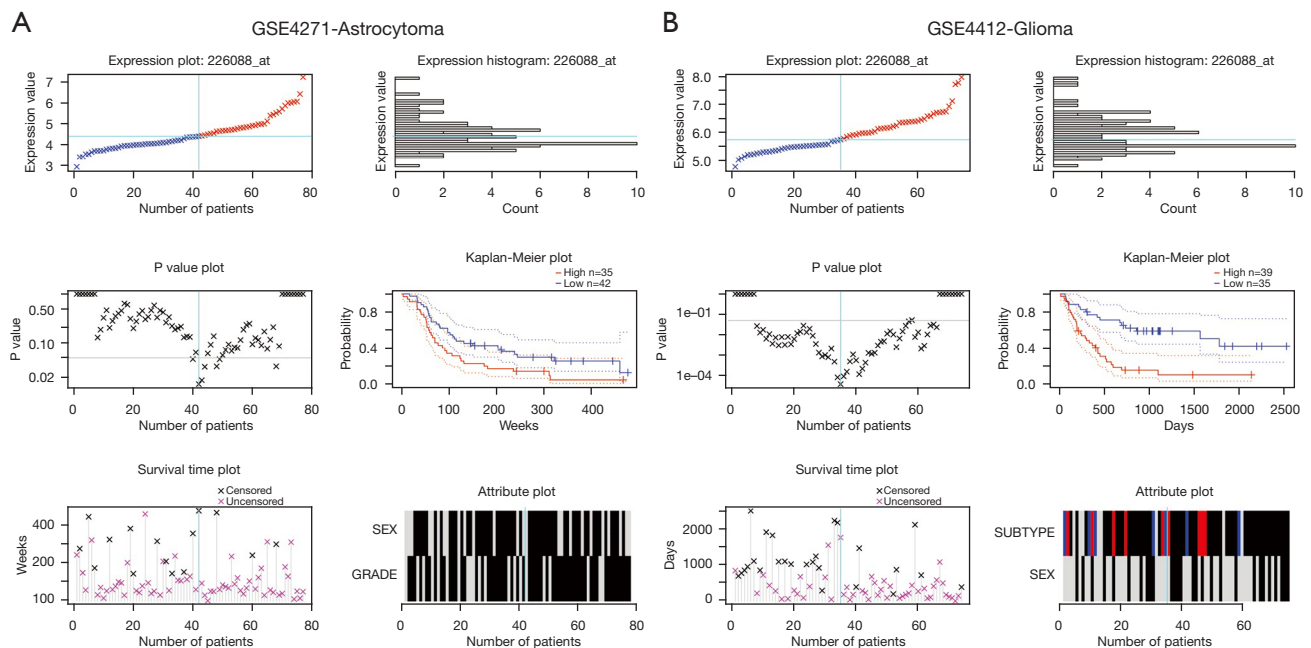
Figure 3 Prognostic significance of ZDHHC12 in the GSE4271 (A) and GSE4412 (B) datasets. ZDHHC, zinc finger DHHC-type palmitoyltransferase.

decreased in GBM samples, compared with the other glioma histology types, such as oligodendroglioma and astrocytoma, among others (*Figure 5A*). Similarly, *ZDHHC12* methylation also decreased in advanced WHO grades (II and III) (*Figure 5B*) and IDH-WT groups (*Figure 5C*), suggesting that the methylation score of *ZDHHC12* is correlated with glioma progression. Next, by using the methylation level as a marker, we examined the prognostic significance of *ZDHHC12* in glioma. Notably, glioma patients with higher *ZDHHC12* methylation had a worse overall survival time (*Figure 5D*), implying that *ZDHHC12* methylation may serve as an unfavorable biomarker. Finally, as *ZDHHC12* exhibited low frequencies of genomic amplifications and deletions in TCGA glioma datasets (*Figure 5E*), we analyzed the relationship between *ZDHHC12* methylation and mRNA levels. Moderate negative correlation (Spearman's =–0.22, and Pearson's =–0.23) was observed between methylation and mRNA expression, suggesting that increased mRNA expression of *ZDHHC12* may be attributable to *ZDHHC12* hypomethylation (*Figure 5F*).

In conclusion, we first identified *ZDHHC12* as an unfavorable prognostic marker in glioma, and loss-of-

function assays suggest that *ZDHHC12* may promote cancer cell growth and migration. Decreased DNA methylation may lead to elevated expression of *ZDHHC12* in glioma.

Discussion

Protein palmitoylation levels are controlled by members of the *DHHC* family of protein palmitoyl transferases (*PATs*) that play oncogenic and tumor-suppressing roles in cancer (12,16,31-33). Therefore, targeting S-palmitoylation or the enzymes responsible for palmitoylation dynamics might be a therapeutic strategy for certain cancers (14,34-37). The role of the *ZDHHC* family in cancer has been widely reported. For example, *ZDHHC5* has been found to regulate cancer stem cells and associated with malignant prognosis in lung adenocarcinoma (38), *ZDHHC9* has been found to induce and stabilize *PD-L1* protein, then drive cancer cells from immune surveillance in breast cancer (39), and the melanocortin-1 receptor palmitoylated by *ZDHHC13* has been found to promote pigmentation and protect against melanoma (17).

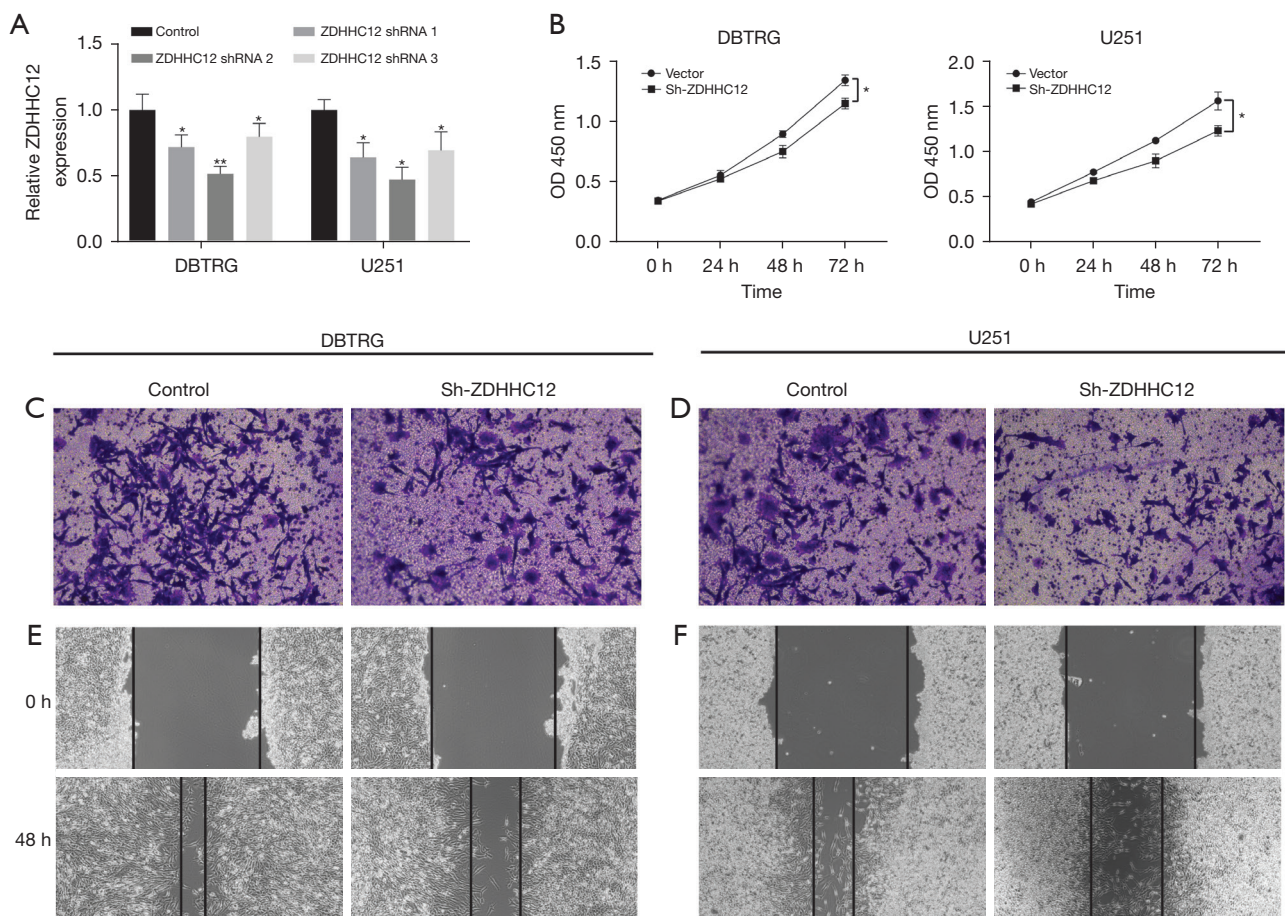In this study, a comprehensive analysis of *ZDHHC12* in pan-cancer found that *ZDHHC12* was upregulated and

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Figure 4 Decreased ZDHHC12 attenuates the proliferation, invasion and migration in glioma cells. (A) The expression of ZDHHC12 was silenced in DBTRG and U251 glioma cells. (B) ZDHHC12 silencing repressed cell growth in DBTRG and U251 glioma cells. (C,D) Transwell assays showing that ZDHHC12 knockdown cells exhibited reduced invasion potential. The cells were stained with 1% crystal violet solution to observe the number of migrated cells. Magnification of image, 100×. (E,F) Wound healing assays demonstrating deceased migration ability in ZDHHC12 silenced glioma cells. ZDHHC, zinc finger DHHC-type palmitoyltransferase. *, $P<0.05$; **, $P<0.01$. Magnification of images, 40×.

could predict poor prognosis in GBM and LGG. Further cellular assays showed that *ZDHHC12* promoted glioma tumorigenesis. In previous studies, the role of *ZDHHC12* has been shown to be implicated in neurological disorders like Huntington's disease, Alzheimer's disease, and schizophrenia (40). In human primary osteoblasts, it has also been found to be involved in 1,25 (OH)D, leading to improved viability and function (41). Furthermore, the knockdown of *ZDHHC12* in ovarian cancer has been shown to significantly inhibit *CLDN3* accurate membrane localization, protein stability, and ovarian cancer cell tumorigenesis (42). To our knowledge, the present study

is the first to reveal the clinical significance and cellular function of *ZDHHC12* in glioma.

Another important finding in this study was the hypomethylation of *ZDHHC12* in glioma. Gene expression is governed by multiple processes on transcriptional and post-transcriptional levels. Given that the genomic alteration frequencies of *ZDHHC12* in glioma were low, we investigated the epigenetic elements influencing increased *ZDHHC12* abundance in high-grade glioma. Notably, decreased *ZDHHC12* methylation was found in advanced glioma groups and IDH WT samples, suggesting that hypomethylation in the *ZDHHC12* gene may occur prior
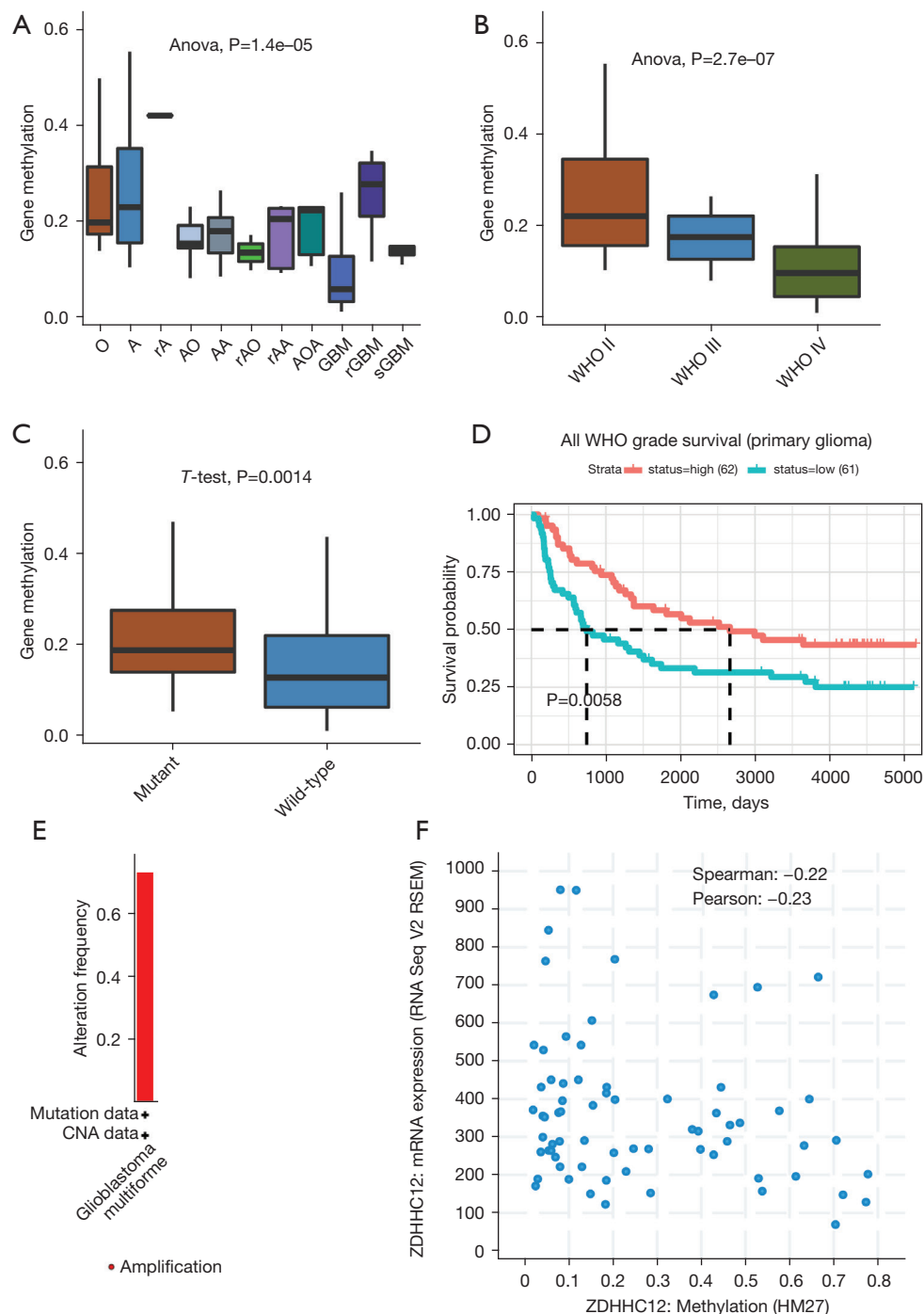
Figure 5 Elevated DNA methylation may contribute to increased expression of ZDHHC12 in glioma. (A) The DNA methylation level of ZDHHC12 significantly decreased in different histological types of glioma tissue (CGGA datasets). (B) The DNA methylation level of ZDHHC12 significantly decreased in glioma tissues with a higher WHO grade (CGGA datasets). (C) The DNA methylation level of ZDHHC12 significantly decreased in IDH wild-type glioma tissues (CGGA datasets). (D) Kaplan-Merrier survival plot of ZDHHC12 methylation level in primary glioma (CGGA datasets). (E) Low amplification was observed in glioma samples (TCGA dataset). (F) Significant negative correlation was observed between ZDHHC12 methylation and expression level (TCGA dataset). ZDHHC, zinc finger DHHC-type palmitoyltransferase; CGGA, Chinese Glioma Genome Atlas; TCGA, the Cancer Genome Atlas, WHO, World Health Organization; IDH, isocitrate dehydrogenase.

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to increased *ZDHHC12* expression, providing an early screening marker for glioma. In addition, the methylation levels of *ZDHHC12* were found to be relevant in predicting glioma survival outcomes and times, which may also benefit patients. The exact regulatory mechanism of DNA methylation with mRNA expression still needs to be studied to explore DNA elements and transcriptional regulons within.

In conclusion, although *in vitro* cellular assays proved that *ZDHHC12* has a role in glioma tumorigenesis and progression, further research is required, including the *in vivo* study of *ZDHHC12* using gain-of-function technologies to assess rescue strategies. Also, the exact substrate of *ZDHHC12* in glioma is not clear, even though claudin 3 (*CLDN3*) emerged as the target of *CLDN3* in ovarian cancer (42). Finally, more in-house glioma samples and related clinical data will need to be collected in the future to assess the performance of *ZDHHC12* in glioma diagnosis and prognosis.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-520/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-520/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-520/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Open Access Statement: This is an Open Access article

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