Comprehensive Analysis to Identify the Vasohibin1 (VASH1) Emerges as a Novel Prognosis Biomarker in High-Risk Low-Grade Glioma

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Abstract

Background: Gliomas are complex and heterogeneous central nervous system tumors, with Low-grade Glioma (LGG) as the most common pathological type. But studies on the predictive effect of a single gene on LGG are limited. VASH1 is an epigenetic regulator with various tumors. However, the role of VASH1 in LGG remains confused. This is the first research focusing on the prognostic value and underlying mechanism of VASH1 in LGG.

Methods: In this research, three independent datasets were used for mRNA-related analysis: two datasets from the TCGA and CGGA (CGGA-mRNA seq 693 and CGGA-mRNA seq 325). We analyzed and screened the clinical significance of VASH1 in overall survival and DSS of various cancers. TIMER and CIBERSORT algorithms were employed to investigate the effect of VASH1 on the tumor microenvironment. GSEA along with GO and KEGG enrichment analyses were conducted to uncover the biological functions of VASH1. In addition, a LGG patient cohort (The First Affiliated Hospital of Xinjiang Medical University) was utilized for analysis of cell infiltration by immunohistochemical, Western-blot, and qPCR; then to verify its function in regulating LGG progression in vitro.

Result: In this study, the results of generalized cancer survival analysis showed that abnormal VASH1 expression was associated with poor prognosis (overall survival (OS) and disease-specific survival (DSS) in patients with adrenal cortical carcinoma (ACC), low-grade glioma (LGG), pancreatic adenocarcinoma (PAAD) and hepatocellular carcinoma (LIHC) (P<0.05). Meanwhile, VASH1 was correlated with the immune invasion, immune score, immune checkpoint, and TBM of the above four tumors, and the correlation between VASH1 expression and LGG was the strongest. In addition, we found that VASH1-mediated changes in gene expression are closely related to cell cycle, P53, Notch, and TGF-β signaling pathways. In addition, immunostaining and RT-PCR were performed on our cohort, and the results showed that VASH1 expression was significantly higher than that of para-cancer tissues (P<0.05). Kaplan-Meier survival analysis results showed that VASH1 was associated with shorter survival (OS) and shorter DFS in high-risk LGG patients (P<0.05). Multivariate Cox analysis showed that high VASH1 expression was an independent risk factor for the prognosis of LGG patients (HR=1.65, P=0.02). Finally, a high level of VASH1 was found in U-251 cell lines by in vitro cell experiments, and the migration and invasion ability of U-251 cells were significantly improved after knockdown of VASH1 (P<0.01), which further confirmed the function of VASH1.

Conclusion: In conclusion, this study preliminarily indicates that VASH1 can be used as a prognostic biomarker and potential therapeutic target for LGG, and has important clinical application value.

Introduction

Low-grade Glioma (LGG) is a common primary intracranial tumor. There is huge heterogeneity among patients with different low-grade gliomas, which results in some low-grade gliomas often progressing to high-grade Glioma, and the prognosis is very poor. More aggressive interventions are needed [1]. Although
many attempts have been made on risk stratification of glioma based on many clinical characteristics, accurate risk stratification and prediction for patients with low-grade glioma are still not possible [2]. Large-scale genomic studies in recent years have shown that the heterogeneity of glioma extends to a deeper molecular level, and it is difficult to accurately serialize the risk of glioma from the macro clinical characteristics, so we need to explore the risk stratification of low-grade glioma from the heterogeneity of molecular level [3]. In recent years, researchers have discovered some promising biomarkers, some emerging markers include isocitrate dehydrogenase (IDH) mutations, co-deletion of 1p and 19q in chromosome arms (1p/19q codeletion), And 0-6 methylguanine-DNA methyltransferase (MGMT) methylation status have been included in 2021 WHO glioma classification to clarify its biological characteristics and guide treatment [4]. However, these widely used biomarkers do not fully accurately elucidate individual variation, and the exploration of glioma risk stratification at the molecular level needs to continue.

As is known to all, LGG patients in good physical condition can obtain a good prognosis after surgery or radiotherapy and chemotherapy, and 50-75% of patients die due to progression and deterioration, so we believe that the characteristics of tumor invasion and metastasis become a key factor affecting the prognosis of patients [5]. VASH1, as a newly discovered angiogenic inhibitor, has been found to play an important biological role in the development and progression of various tumors. VASH1 belongs to a class of angiogenesis regulatory proteins in the angiogenesis inhibitor family, which can inhibit the proliferation of vascular endothelial cells and the formation of neovascularization network and is the first discovered endothelium-derived factor that negatively regulates angiogenesis [6]. VASH1 can be selectively expressed not only in endothelial cells, but also in tumor cells and some immune cells, directly inhibiting the migration, proliferation, endothelial net and promoting apoptosis of endothelial cells, and indirectly inhibiting angiogenesis through negative feedback regulation of vascular endothelial growth factor (VEGF) [7]. In addition, according to literature reports, VASH1 can also enhance stress resistance of endothelial cells by inducing the expressions of superoxide dismutase2 (SOD2) and sirtuin1 (SIRT1) [8]. With the continuous progress of research on VASH1, more and more scholars at home and abroad have developed the special biological characteristics of VASH1 and applied them to clinical treatment as a tumor target. At present, much literature has discussed the clinical role of VASH1 in gastric cancer [9], ovarian cancer [10], colorectal cancer [11], esophageal cancer [12], prostate cancer [13], and non-small cell lung cancer [14]. There are few reports on the role of VASH1 in LGG. Further studies on the molecular mechanism of VASH1 affecting the genesis and development of LGG will help to identify molecular targets for tumor therapy and provide clues for the development of new and more powerful anti-tumor tools.

In the present study, we first discovered that VASH1 mRNA expression was up-regulated in LGG samples in the TCGA databases. We used TIMER and ESTIMATE to further understand the correlation between VASH1 and infiltrating immune cells in LGG. In addition, we conducted GO and KEGG pathway enrichment analysis on genes related to VASH1 expression in LGG through GSEA, to determine the potential mechanism of VASH1 in the occurrence and development of LGG. Therefore, in our follow-up study, the expression of VASH1 in our cohort study was determined by immunohistochemistry, and a
clinical prediction model was constructed. Finally, VASH1 was tested in vitro. Our study provides a new therapeutic target and Prognostic method for patients with LGG.

**Method And Materials**

**Data collection and preprocessing**

From the cancer genome atlas (TCGA) database (https://portal.gdc.cancer.gov/) and genotype-organization express project (GTEX) database (https://gtexportal.org/) download 33 kinds of tumor gene expression data and normal tissue and tumor tissue Clinical information. Transcriptome (fragments per kilobase million, FPKM), somatic mutation data, copy number variation (CNV), and clinical phenotype data for LGG were downloaded from the TCGA database. Corresponding to heavy annotation in gene chip RNA probe, we download the appropriate RNA genome sequence information and data from the GENECODE database (https://www.gencodegenes.org/human/). The RNA expression profile of the reannotated microarray was constructed by matching the sequence information of the probe with that of RNA. The human genome annotation file (GRCh38/hg38) from the UCSC database (http://hgdownload.cse.ucsc.edu/) to download. In addition, the department of cancer cells encyclopedia (CCLE) database downloaded 21 tumor cell lines (such as breast, thyroid, and uterine) information (https://portals.broadinstitute.org/). Finally, based on the FTO expression levels of 33 cancers, univariate survival analysis was used to study the prognosis of patients in terms of overall survival (OS) and disease-specific survival (DSS). Kaplan-Meier curves and forest maps were visualized for cancer with significant statistical differences.

**Correlation between tumor immune cell infiltration and VASH1 gene expression**

Tumor immune to assess resource (TIMER, https://cistrome.shinyapps.io/timer/) is a comprehensive database, can be systematically analyzed of various types of cancer of the immune infiltrating [15]. Spearman correlation was used to estimate the correlation between VASH1 expression and levels of 47 immune checkpoint genes in tumor immune infiltrating cells (CD4 + T cells, B cells, CD8 + T cells, macrophages, neutrophils, and dendritic cells) in 33 cancers. In addition, association analysis of VASH1 with stroma scores for multiple cancers was evaluated by software estimation. At the same time, the relationship between gene expression and immune score was analyzed in 33 tumor samples using the R package ESTIMATE. Secondly, TMB is defined as the total number of somatic gene coding mutations existing in tumor tissues, such as deletion errors or gene insertions [16]. MSI refers to a strongly mutated phenotype caused by loss of DNA mismatch repair activity [17]. Both TMB and MSI are potential predictive biomarkers of immune checkpoint therapy. We extracted TMB and MSI data from the TCGA database. Spearman analysis was used to estimate the correlation between VASH1 expression level and TMB or MSI status.

**VASH1-related gene enrichment analysis**
We first searched the STRING (https://string-db.org/)[18] and GeneMANIA (https://genemania.org/).[19] website using the query of a single protein name (“VASH1”) and organism (“Homo sapiens”). Subsequently, we set the following main parameters: minimum required interaction score [“Low confidence (0.150)”], meaning of network edges (“evidence”), max number of interactors to show (“no more than 50 interactors” in 1st shell) and active interaction sources (“experiments”). Finally, the available experimentally determined VASH1-binding proteins were obtained.

**Gene Oncology (GO) Annotation and KEGG Pathway Enrichment Analysis**

Differentially expressed genes (DEGs) between two groups were screened by using the "DESeq2" package in R software according to the thresholds of |log2FoldChange| > 1 and adjusted p< 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to annotate the biological functions of DEGs and VASH1-related genes through "clusterProfiler" package. With the annotated gene sets in "h.all.v7.4.symbols.gmt" chosen as the reference gene sets, gene set enrichment analysis (GSEA) was conducted to investigate the potential regulatory mechanisms of VASH1.

**External Validation of genes in the VASH1 mRNA risk score Model**

The feature model genes were verified by CGGA mRNA seq-693 and CGGA mRNA seq-325 in The Chinese Glioma Genome Atlas (CGGA) database and GSE16011 in the Gene Expression Summary Database (GEO). The same formula was used to calculate the risk score, and Boxplot was used to compare the gene expression of different genders, tumor stage (1 and 2), tumor types (primary and recurrent), and VASH1 expression status.

**Management of tissue specimens**

All LGG patients and tissue samples involved in this study were from the neurosurgery sample bank of the First Affiliated Hospital of Xinjiang Medical University. 204 cases of intracranial tumor resection in the First Affiliated Hospital of Xinjiang Medical University from January 2013 to December 2019 were randomly selected. Excluding meningioma, patients who received preoperative chemoradiotherapy, and patients with incomplete follow-up information, the remaining 94 LGG, and 68 GBM patients underwent paraffin-embedded surgical tissue samples for immunohistochemical staining. Postoperative intracranial tumors were independently diagnosed as low-grade glioma (grade I(12 cases), grade II(52 cases), and grade III(31 cases)) and high-grade glioma (grade IV(68 cases) by 2 pathologists according to WHO grading standards. Basic clinical data and standard clinical follow-up of 94 patients were included. The follow-up period of the study was up to the end of December 2020. In addition, we also collected 16 fresh pathological specimens and adjacent normal brain tissue samples from 8 LGG patients who underwent surgical treatment in the First Affiliated Hospital of Xinjiang Medical University from January 2021 to September 2021 for PCR detection. The medical Research Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University approved the study. According to the Declaration of Helsinki, the
samples and case data used in this study were approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

The expression of VASH1 was detected by immunohistochemistry

Immunohistochemical analysis, LGG pathological tissue was embedded in paraffin and sectioned on a 4μm microtome. Slices were placed on slides and dehydrated with different concentrations of alcohol solutions (75%, 80%, 90%, 95%, 100%) at different times and cleaned with xylene. A two-step indirect immunohistochemical staining was used in this study. Rabbit antibodies against human VASH1 (Abcam, United Kingdom) were diluted to 1:200 and 1:250, respectively. Antibody staining was performed overnight at 4°C. The reaction of 3,3'-Diaminobenzoaniline (DAB) substrate-chromogen with peroxidase-conjugated secondary antibody was used to fix paraffin-embedded tissue sections with formalin. DAB can react with the slices to produce brown products insoluble in ethanol and xylene at the antigen site. The sections were rinsed with phosphate buffer solution 3 times, and then the expression of target proteins in the tissues was observed with a light microscope at 200 times magnification. Five high-power fields were randomly selected, 500 cells were counted, and the percentage of immunohistochemical cells in the total number of cells was calculated. The percentage ≥ 50% was the high expression, and <50% was the low expression. According to the expression level of VASH1, the cells were divided into a high expression group of VASH1 and a low expression group of VASH1. Finally, we divided LGG patients into high expression groups and low expression groups according to the median value of VASH1 as the risk cutoff point. The Kaplan-Meier method was used to draw survival curves of the two groups to predict their prognostic significance in OS and DFS, and P<0.05 indicated a significant statistical difference.

Construction and validation of gene prognostic nomogram

To test whether VASH1 is a prognostic risk factor independent of other clinicopathological features, we performed univariate and multivariate Cox regression analyses using R software for each variable in our 94 LGG cohort. Statistical significance was set at P<0.05. Will the single factor and multiple factors Cox proportional hazards regression analysis of all independent prognostic parameters build a composite nomogram, according to the characteristics of the different variables to draw a straight and level to determine the points of each variable, by taking the point of all variables to calculate the sum of the total points of each patient, and the distribution of its normalization to 0 to 100. The OS values of patients with low-grade glioma at 3 years were calculated between the total score and each prognostic axis. We used the "rms" R software package to draw calibration diagrams to verify the performance of the row diagram in the 94 LGG queues we were able to collect. In addition, we plotted tROC curves to assess the predictive accuracy of independent prognostic parameters using the R package "survivalROC".

Cell line and cell culture

All cell lines including U-87, U-251, and A-172 were purchased from the Cell Bank of Chinese Academy of Sciences (China). All cell lines were cultured in DMEM medium with 10% newborn bovine serum,
supplemented with penicillin 100u/mL and streptomycin 100μg/mL, in a 5% CO2 incubator with saturated humidity and 37°C constant temperature.

RNA Extraction and qRT-PCR

Total RNA was extracted from tissues and cells according to TRIzol reagent instructions, and cDNA reverse transcription was performed according to the instructions of the RT-PCR kit (Invitrogen, United States). GAPDH and VASH1 expression levels were detected by qRT-PCR using SYBR Green qPCR Master Mix (High ROX) (Servicebio, Wuhan, China). Results The expression level of GAPDH was taken as standard. Results The expression level of GAPDH was taken as standard. The PCR primer sequence was designed and synthesized by Servicebio (Wuhan). GAPDH-F: 5′-GGAAGCTTGTCATCAATGGAAATC-3′, GAPDH-R: 5′-TGATGACCCTTTTGCTCCC-3′, VASH1-F: 5′-GTTTTGGAGACCAGCGAAGGAA-3′, VASH1-R: 5′-ACAGGTGTAGACGGCTGGAAC-3′. The relative expression levels of VASH1 were quantitatively calculated by the 2^(-ΔΔCT) method. The amplification reaction included the following steps: Pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s over 40 cycles, and extension at 60°C for 30 s. From 65°C to 95°C, the fluorescence signal was collected every 0.3°C.

Western-Blot

Collecting cells, cell lysis solution treatment, the supernatant, centrifuge after Coomassie brilliant blue method to determine protein concentration, polypropylene phthalic amide gel electrophoresis, The protein electricity is transferred to the nitrate fiber membrane, combined with VASH1 antibody (1:10 dilution degrees 00, Abcam), combined with horseradish peroxide enzyme after two combinations, ECL method after color photograph. Finally, the gray values of each band were determined by Image-Pro Plus 4.5 Image analysis system to reflect the expression level of VASH1 protein.

Construction and transfection of lentivirus

The relevant information of the VASH1 gene was searched through the Genbank database, and the online design software of Ambion was applied to select the human VASH1 gene (gene serial number: The sequence of siRNA was 5′-CGACCGGaAGaAGGATGTTC-3′ at 1307-1331 of cDNA. BLAST search confirmed no homology with known human gene sequences other than VASH1. DNA Oligo of VASH1 shRNA was designed and synthesized, double-stranded DNA was formed by annealing, Hpal and EcoRI were digested and ligated, and transformed into PGCL-GFP expression plasmid of Ecoli DH5a. Recombinant positive clones were selected for PCR and sent for sequencing identification (Shanghai genechem Technology). 293T cells were co-transfected with pgCL-GFP vector 20μg, pHelper 1.0 vector 15μg, pHelper 2.0 vector 10μg. 8h after transfection, the cells were replaced with a complete culture medium. After 48 h of culture, cell supernatant rich in lentivirus particles was collected and stored for future use.

Transwell assay
Transwell cell was placed in a 24-well plate, the substrate glue was added to the Transwell cell, and the complete culture medium was added to the substrate. After digestion and resuspension, the cells of each group were inoculated in the upper chamber of Transwell, and the number of cells was 3×10⁴. After 48 h culture, the cells that did not invade the subchamber were washed away. Then it was fixed with 4% polymethanol and stained with 0.1% crystal violet for 20 min. The number of cells invading the subcompartment in each field was counted under an inverted microscope.

**Wound-healing assay**

The cells in each group were digested by trypsin and inoculated into 6-well plates with 1×10⁶ cells in each well. Then the cells were cultured at 37°C, 5% CO₂, and 100% relative humidity until the cells reached about 90% confluence. Then make scratches from top to bottom with 200μL pipette tip and wash away the scratched cells with PBS buffer. Then the culture was continued for 24 h under the same conditions. The single-layer images were observed by an inverted microscope, and the migration ability of cells was analyzed by measuring the movement distance of the cell front and the width of the scratch.

**Statistical Analysis**

All bioinformatics and clinical characters analyses were performed in R version 4.0.3, and all experimental data analysis was carried out in GraphPad Prism 9. The significance of the differences between the groups was assessed by the Student’s t-test. The Chi-square test or Fisher test was used for categorical variables, and the Wilcoxon test was used for continuous data. Survival differences were calculated using K-M and logarithmic rank tests. In addition, the use of interactive gene expression profile analysis GEPIA2 [http://gepia2.cancer-pku.cn/](http://gepia2.cancer-pku.cn/) and UALCAN [http://ualcan.path.uab.edu/index.html](http://ualcan.path.uab.edu/index.html), Different expression analyses were further performed on LGG samples from TCGA and normal samples from matched TCGA normal and genotype-tissue expression (GTEx) data. P<0.05 was statistically significant.

**Results**

**Expression and Prognostic Potential of FTO Was Altered in Human Pan-Cancer**

First, we used AVSH1 expression levels in cancer and normal tissue samples from the TCGA database. Given the limited number of normal samples in the TCGA database, we integrated the expression of VASH1 in cancer and normal tissue samples from the GTEx and TCGA databases and found that compared with the GTEx normal control group, VASH1 was significantly higher expressed in various types of cancer, including ACC, BLCA, BRCA, CHOL, ESCA, GBM, HNSC, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, OV, PAAD, PRAD, SKCM, STAD, TGCT THCA, UCEC (Fig. 1A)(Table.1). Meanwhile, we used the CCLE database to calculate VASH1 expression in different tumor cell lines. The results showed that VASH1 showed different expression levels in different tumor cell lines (Fig.1B). In short, the present results suggest that VASH1 is expressed differentially in multiple cancers. To explore the relationship
between VASH1 and clinical outcomes in 33 cancer patients, a univariate analysis was performed using the TCGA dataset. Forest map showed that VASH1 had significant effects on OS and DSS of specific tumor types in 33 cancers evaluated (Fig1C,1D), where KIRC, LGG, there were significant statistical differences in PAAD and LIHC cancers (P<0.05), and the results were visualized using Venny diagram (Supplementary Fig.1).

Secondly, Kaplan-Meier survival analysis was performed for the four selected cancers, and the results showed that high expression of VASH1 indicated a good correlation between KIRC and OS in PAAD (P<0.001). The high expression of VASH1 was significantly correlated with bad OS in LGG (P=0.015) and LIHC (P<0.001) (Fig.1E). Next, we found that high expression of VASH1 significantly prolonged DSS in KIRC (P<0.0001), while high expression of VASH1 was significantly correlated with shorter DSS of LGG (P=0.016), LIHC (P=0.024), and PAAD (P=0.013) (Fig.1F). Overall, these results suggest that VASH1 expression is significantly associated with patient prognosis, especially in patients with KIRC, LGG, LIHC, and PAAD.

**VASH1 Was Associated with Tumor Immune Infiltration, Immune Checkpoint Biomarkers, and TMB in Multiple Cancers**

Based on the TIMER database, we analyzed the immune cell infiltration levels of the four cancers (KIRC, LGG, PAAD, and LIHC) screened above, As shown in Figure 2A. Therefore, we explored whether VASH1 expression is associated with the level of immune invasion in these four cancers. We found that the expression level of VASH1 was significantly correlated with the degree of immune infiltrating cells in KIRC, LGG, PAAD, and LIHC, ranking LGG, KIRC, LIHC, and PAAD in sequence (Fig.2B). In addition, we used R package estimation to assess the stroma score for each tumor sample. VASH1 expression was positively correlated with matrix score for LGG (R= 0.165, P <0.020), LIHC (R=0.03, P=0.637) and KIRC (R= 0.027, P=0.596) (Fl.2C).

Since immunotherapy is a key therapy for tumor reduction and eradication, the relationship between VASH1 expression and 47 immune checkpoint gene expression was further analyzed. Interestingly, the analysis showed that VASH1 expression was positively correlated with immune checkpoint genes common in a variety of cancers, especially in LGG (Fig.2D). VASH1 is important for the complex pattern of modulating tumor immune responses by modulating immune checkpoint genes. In addition, tumor cells with high TMB have high levels of neoantigens, which are thought to stimulate the anti-tumor response of lymphocytes and help the immune system recognize tumors. Our analysis showed that VASH1 expression was positively correlated with TMB in LGG, THYM, UCEC, BLCA, BRCA, CESC, COAD, LUAD, LUSC, SKCM, and STAD. In contrast, VASH1 expression was negatively correlated with PRAD and THCA (Fig.2E). Taken together, our study suggests that VASH1 may play an important role in tumor immune response.

**TME Characteristics of the TCGA Subtype and LGG Somatic Genome**
We used R software (Version 3.6.4) to calculate the expression differences of the VASH1 gene in the genome and non-mutant samples in each tumor, and Wilcoxon Rank and Signed Rank Tests were used for significance analysis of the differences. We found that there were significant expression differences in GBM-LGG, LGG, KIPAN, MESO, and SKCM samples (Fig.3A). Combined with the above analysis, we believed that VASH1 expression level was closely correlated with LGG. At present, immune checkpoint blocking (ICB) therapy has been applied in a variety of tumor diseases, improving the overall survival rate of patients. Many studies have shown that this tumor mutation load (TMB) can be used to predict the efficacy of ICB, and it has become a biomarker for various cancer types to identify patients who will benefit from immunotherapy. Based on the clinical significance of TMB in immunotherapy, we further explored the internal relationship between TMB and VASH1 expression to clarify the genetic information related to VASH1. Correlation analysis showed that TMB was positively correlated with VASH1 (R=0.095, P=0.035, Fig.3B). We found the optimal threshold through the “SurvMiner” R package and divided the patients into the high group and low group, and conducted a box plot (P=0.008, Fig.3C). The expression levels of TMB and VASH1 in patients were combined for analysis. The results showed that patients with high VASH1 and high TMB had the worst prognosis, while patients with low VASH1 expression and low TMB had the best prognosis (P <0.001, Fig.3D).

We obtained the LGG driver gene and evaluated somatic mutations in VASH1 patients with different expression levels. Fig.3E and 3F respectively showed the mutation distribution of the 20 driver genes with the highest change frequency in the high and low VASH1 groups. These results may provide new directions for the study of immunotherapy mechanisms, gene mutations, and molecular mechanisms of VASH1 in LGG.

The Aberrant Expression and Prognostic Value of VASH1 in LGG Patients

miRNA expression levels in RNA-seq data and corresponding clinical data from 510 LGG samples obtained from TCGA. To explore the prognostic significance of VASH1 in LGG patients, we divided LGG patients into high VASH1 high expression group and low VASH1 low expression group based on the optimal cutoff point calculation of the expression group through "Survival" and "SurvMiner" software package. VASH1 expression distribution and survival status of LGG are shown in FIG. 4A. In addition, we verified online GPEIA and UALCAN data and found that VASH1 expression was significantly different in LGG (n=518) patients compared to normal tissue (n=207) (Fig.4B, 4C). At the same time, k-M survival analysis was performed, and the results showed that high expression of VASH1 in GPEIA and UALCAN was associated with poor prognosis. Further above results were obtained (Fig.4D, 4E). Finally, we analyzed the correlation between clinical prognostic factors and VASH1 expression levels at different time points of LGG patients by ROC and tROC curves, and the results showed that VASH1 expression had a certain predictive ability, and LGG patients with high VASH1 expression had poor clinical prognosis in 3-year OS (Fig.4F, 4H). In conclusion, VASH1 may be a potential prognostic biomarker for LGG patients.
The biological function of VASH1 in LGG

To further explore the potential function of VASH1 in patients with LGG, differentially expressed genes were analyzed between the groups with high VASH1 expression and low VASH2 expression. The most important GO terms for biological processes (BP), cellular composition (CC), and molecular function (MF), as well as KEGG pathways, were analyzed to reveal the underlying biological function of differentially expressed genes, and the top 30 significant GO terms were selected. It is mainly involved in potassium channel activity, DNA binding, cholesterol biosynthetic process, and labyrinthine layer blood vessel development, negative regulation of blood vessel endothelial cell migration and stabilization of membrane potential are related to angiogenesis regulation, cholesterol metabolism, and microtubule formation (Fig. 5A). Meanwhile, KEGG pathway analysis showed that the cholesterol metabolism and folate biosynthesis pathways of VASH1 were mainly involved (Fig. 5B). These signaling pathways are related to core biological carcinogenic processes, most of which involve the regulation of carcinogenic activation pathways, cell cycle, angiogenesis, and immune cell infiltration. Then, we constructed different protein and gene co-expression networks associated with VASH1 expression through the String and GeneMANIA online databases (Fig. 5C, 5D). In addition, to further explore the correlation between VASH1 expression level and prognosis of LGG, through GSEA, we found that VASH1 expression was closely related to glioma, ECM-receptor-interaction, cell cycle, TGF-β, P53, and Notch signaling pathways (Fig. 5E). This may provide a new molecular mechanism for exploring the genesis and development of LGG.

Verification of the VASH1 in two external independent LGG datasets

To cross-platform validation of VASH1 expression level and clinical prognosis of LGG in other independent data sets from different platforms, VASH1 was covered by CGGA mRNA seq-693 and CGGA mRNA seq-325, with a large sample size and common clinicopathological features. Therefore, we selected CGGA mRNA seq-693 and CGGA mRNA seq-325 data to study the correlation between VASH1 expression level and gender, age, WHO grade, chemotherapy status, IDH status, 1p/19q chromosome co-deletion, and tumor type of LGG patients. In the CGGA mRNA seq-693 dataset VASH1 was significantly correlated with gender and tumor type (P<0.05) (Fig. 6A, 6B). In addition, the correlation with these indicators was also observed in VASH1 in the CGGA mRNA seq-325 dataset. The results showed that VASH1 was closely correlated with WHO grade in low-grade gliomas (P<0.05) (Fig. 6C). At the same time, survival analysis was performed on LGG patients with complete clinical data from CGGA mRNA seq-693 and CGGA mRNA seq-325 data sets respectively, and the results showed that VASH1 expression was significantly correlated with the prognosis of LGG patients, that is, high VASH1 expression was correlated with poor prognosis (HR=1.39, P=0.04, HR=1.42, P=0.02) (Fig. 6D, 6E). This result further confirms our above results.
Experimental results confirmed that the expression level of VASH1 in LGG tissues was significantly higher than that in normal tissues

Then, 94 cases of LGG and 68 cases of GBM hospitalized in the Neurosurgery Department of the First Affiliated Hospital of Xinjiang Medical University from January 2014 to December 2019 were collected for postoperative paraffin specimens for pathological sections, and the expression of VASH1 in tumor tissues and adjacent normal tissues was detected by immunohistochemistry. According to the observation of two pathologists (with unknown patient information), immunohistochemical results showed that VASH1 protein was mainly expressed in the nucleus and cytoplasm of glioma cells and endothelial cells and showed brown positive expression (Fig.7A). At the same time, most of VASH1 was positively expressed in LGG tissues (57/94, 60.6%), and the expression level was statistically significant (P<0.05) compared with normal adjacent tissues and GBM tissues (Fig.7B). Subsequently, to continue to explore the expression of VASH1 in freshly frozen LGG tissues and adjacent tissues, we randomly selected 8 patients who underwent glioma resection in the First Affiliated Hospital of Xinjiang Medical University from January 2021 to September 2021 and considered that LGG tissues and corresponding adjacent normal tissues after surgery were taken. Total RNA proteins were extracted from tumor tissues and normal tissues, and the relative expression level of VASH1 mRNA was detected by real-time PCR, and the relative expression level of VASH1 was calculated by the deta CT method. The results showed that the expression of VASH1 mRNA in LGG in most specimens was significantly higher than that in the corresponding normal brain tissue (P<0.01) (Fig.7C,7D).

Relationship between VASH1 expression and pathological parameters and prognosis of LGG patients

First, according to the expression level of VASH1, we analyzed the clinicopathological parameters and prognostic factors of 94 LGG patients in our hospital and found that there was no statistically significant difference in age, gender, histology, tumor size, location, and KPS score between the two groups. However, tumor recurrence, WHO grade, epilepsy, and IDH1 wild type were significantly correlated with VASH1 expression (P<0.05) (Table.2). We found that VASH1 expression was closely related to the grade of LGG malignancy and tumor recurrence, so does VASH1 expression affect the prognosis of LGG patients? In this regard, we systematically followed up the postoperative prognosis of each LGG patient and analyzed the overall survival rate (OS) and Disease-Free Survival (DFS) of the patient according to our follow-up results. The results showed that the 3-year OS and DFS of patients with high VASH1 expression group were significantly lower than those of patients with low VASH1 expression (P<0.05). Then, the K-M survival curve was drawn based on overall survival time and disease-free survival time, and the log-rank test was used to analyze the differences between the two groups. The results showed that the survival time of patients with a high VASH1 expression group was significantly lower than that of patients with a low VASH1 expression group (P=0.0352) (Fig.7E). The disease-free survival time of patients with a high VASH1 expression group was significantly lower than that of patients with a high VASH1 expression group (P=0.0071) (Fig.7F).
This Nomogram should be established to predict the prognosis of LGG for high VASH1 expression

Pathological features and VASH1 expression affecting the prognosis of LGG patients were included in Cox proportional risk regression models for univariate and multivariate regression analyses, respectively. Univariate results are shown in Figure 8A. For LGG patients, tumor recurrence (HR=3.70, P<0.001), WHO grade (HR=2.81, P<0.001) and high VASH1 expression (HR=1.54, P=0.03) are the single prognostic risk factors for OS (Table 3). Further multi-factor analysis shows that: Tumor recurrence (HR=3.47, P<0.001), WHO grade (HR=3.13, P<0.001), and high VASH1 expression (HR=1.65, P=0.02) were independent risk factors for OS in LGG patients (Fig.8B). There was no correlation between age, gender, and IDH1 type and prognosis of LGG (Table 3). In addition, a Nomogram model for LGG patients with age, gender, tumor type, WHO grade, IDH1 type, and VASH1 expression was established using R software based on relevant research results at home and abroad. By constructing nomograms, the prognostic factors can be applied clinically to predict the 3-year survival rate of patients (Fig.8C). The calibration diagram (Fig.8D) shows that the topograph has a good prediction effect. Compared with the risk scoring model, the prediction performance of the line chart is significantly improved. Finally, according to the expression of VASH1 in 94 LGG patients, we conducted ROC curve analysis with time changes for 3 years and obtained AUC values of 0.82 (95% CI: 0.93-0.72) (Fig.8E). Therefore, it is believed that the high expression of VASH1 provides a new risk molecular stratification for the diagnosis of high-risk LGG.

Human glioma cell lines with VASH1 knockdown were constructed

To further understand the effect of VASH1 expression on the biological behavior of LGG, we constructed glioma cell lines with VASH1 knockdown for functional experiments. Firstly, we used real-time PCR and Western-blot to detect the expression of VASH1 mRNA and protein in common human glioma cell lines (A-172, U-251, and U-87) and found that the expression of VASH1 mRNA and protein was the highest in the U-251 cell line. Therefore, we planned to select U-251 cells to knock down VASH1 for subsequent VASH1 expression and function experiments (Fig.9A, 9B, 9C). In this experiment, VASH1 short hairpin RNA designed by genechem (Shang Hai) and control nonfunctional shRNA plasmid lentivirus were transfected into U-251 cells to construct U-251Si-VASH1, U-251Si-NC, and their control cell line U-251Normal. In order to determine the expression of VASH1 after infection, the mRNA and protein expression of VASH1 in U-251Si-VASH1 cells were detected by real-time PCR and Western-blot, and the results confirmed that the mRNA and protein expression of VASH1 in U-251Si-VASH1 cells were significantly decreased compared with the control group and normal U-251 cell line. The difference was statistically significant (P<0.05) (Fig.9E, 9F, 9G). The above experiments confirmed that the interference effect of the designed knockout plasmid was satisfactory, so we carried out subsequent functional experiments on U-251Si-VASH1, U-251Si-NC, and U-251Normal cell lines.

Effects of VASH1 on migration and invasion of U-251 cells
The invasion and migration ability of tumor cells is a key characteristic affecting tumor invasion and recurrence, and current studies have shown that the high expression of VASH1 is significantly correlated with LGG WHO grade and recurrence. Therefore, we further studied the effect of VASH1 on the migration and invasion ability of glioma cells through in vitro experiments. First, we used a scratch test to detect the migration ability of abnormal VASH1 expression to U-251 cells. The results showed that u-251\textsuperscript{Si-VASH1}, u-251\textsuperscript{Si-NC}, and U-251\textsuperscript{Normal} cells were scratched 48 hours after VASH1 expression was interfered with by monolayers fusion. The speed of scratch healing in U-251\textsuperscript{Si-VASH1} was significantly faster than that in U-251\textsuperscript{Si-NC} and U-251\textsuperscript{Normal} cell groups (P<0.01) (Fig.9H,9I). Then, we carried out the Tranwell invasion experiment on the above three groups of cell lines. U-251\textsuperscript{Si-VASH1}, U-251\textsuperscript{Si-NC}, and U-251\textsuperscript{Normal} cells were inoculated in the upper chamber of the Transwell chamber, and a complete medium was added in the lower chamber. After 12-24h incubation, It was observed that the number of cells passing through the bottom of the Transwell chamber in U-251\textsuperscript{Si-VASH1} cells was significantly higher than that in the other two groups (P<0.001), while there was no significant difference between U-251\textsuperscript{Si-NC} and U-251\textsuperscript{Normal} cells (P>0.05) (Fig.9J, 9K).

Discussion

Glioma is the most common intracranial primary tumor, accounting for 81% of central nervous system malignancies. Low-grade glioma (LGG) is a group of heterogeneous tumors, accounting for nearly 20% of all primary brain tumors, and median overall survival (median overall survival, mOS) (5.6 ~ 13.3) years [20]. Although surgery, radiotherapy, and chemotherapy are the main treatment modalities for LGG, the optimal combination therapy for a specific patient has not been determined based on individual symptoms and the risk of toxicity caused by treatment [21]. Although a number of clinical trials have shown that high-risk LGG shows positive effects in postoperative radiotherapy and chemotherapy, there are still many debates [22]. Although risk stratification for high-risk LGG has been carried out clinically and molecularly, various stratification standards have their advantages and disadvantages, and their role in the prognosis of LGG is still controversial [23]. In this study, we further explored the prognostic factors of LGG patients. We first found through TCGA, GTEx, CCLE, GENECODE single-cell RNA-seq datasets, and our cohort that VASH1 expression was significantly up-regulated in LGG tumor tissues compared with normal tissues. Further Cox proportional risk regression model showed that VASH1 expression level had an independent prognostic value for LGG. The results confirmed the correlation between VASH1 expression level and the progression and prognosis of LGG.

At present, a large number of studies have confirmed that VASH1 expression level is correlated with the prognosis of various solid tumors. Yan et al. [24] reported A positive correlation between VASH1 expression level and VEGF-A and microvascular density (MVD) in colon cancer tissues. VASH1 expression was significantly positively correlated with pathological TNM stage, tumor stromal invasion, lymph node involvement, distant metastasis, and shorter survival. After follow-up, Cox proportional risk regression model analysis showed that VASH1 and lymph node metastasis were independent risk factors for the prognosis of colon cancer patients, respectively. It is noteworthy that, in contrast to the colon cancer
study, Zhao et al. [25] showed that high VASH1 expression was associated with a better prognosis in renal cell carcinoma. This further indicates that VASH1 has different types of regulatory effects in different types of tumors and tissues. We analyzed the expression levels of VASH1 in different types of cancer and cells through the TCGA database, and the results showed that VASH1 was strongly correlated with LGG. Compared with normal tissues, VASH1 expression was significantly increased in LGG, and its high expression could be used as an independent risk factor for the prognosis of LGG. Then, immunohistochemistry and real-time PCR results were performed on LGG, GBM, and normal tissues to further confirm that the expression level of VASH1 in LGG was significantly higher than that in normal tissues, and it was mainly expressed in tumor cells and endothelial cells. Meanwhile, the VASH1 expression level was positively correlated with tumor recurrence, WHO grade, epilepsy, and IDH1 wild type. These results further confirmed the strong specificity of VASH1 in gliomas, and further reflected the progression and deterioration of tumors.

Tumor-infiltrating immune cells are closely related to tumorigenesis, angiogenesis, and tumor cell growth, thus regulating the number and differentiation of immune cells [26]. There is evidence that tumor progression may be caused by the escape of cancer cells from host immune monitoring [27]. Therefore, elucidating the infiltrating immune cells in TME may help elucidate the underlying mechanisms of VASH1 in LGG. We found that the proportion of anti-tumor immune cells was higher in the high expression group, and VASH1 was positively correlated with B cells, CD4+T cells, macrophages, neutrophils, and dendritic cells. In addition, we also found differential genes mediated by VASH1 and abundant immune pathways. VASH1 is positively correlated with KLHL-1, PAXX, and CXXC4 in LGG, and is related to the Natural killer cell-mediated cytotoxicity pathway. They are associated with inhibition of NK cells and macrophage infiltration, inducing immune escape of tumor cells, thereby promoting tumor growth. At the same time, we found that VASH1 was positively correlated with various immune checkpoints in LGG, and VASH1 mutations in LGG immune microenvironment can promote tumor growth and lead to poor prognosis. Therefore, VASH1 alone or in combination with other targets may serve as a potential biomarker for immunotherapy.

So far, the molecular mechanism of VASH1 in tumor genesis and development is not completely clear [28]. It has been reported that VASH1 is transported extracellularly by binding with a molecular chaperone, and then binds to vascular growth factor receptor-2 (VEGFR-2) on the surface of endothelial cells to inhibit the activation of downstream pathways after VEGFR-2 binding, thus inhibiting angiogenesis [29]. Ninomiya et al. [30] performed immunostaining on postoperative surgical specimens of esophageal cancer patients and found that VASH1 and VASH2 expressions were related to tumor progression and prognosis, among which VASH1 positive esophageal cancer patients had a poor prognosis. However, due to the lack of a typical secretory signaling sequence for these two regulatory factors, VASH1 needs to bind to a small Vasohibin-binding protein (SVBP) in order to be secreted effectively and increase the stability of protein structure while promoting the secretion of VASH2. It also plays an important role in the regulation of tumor angiogenesis [31]. In addition, studies have reported that after endothelial cells interfere with the expression of VASH1, high expression of VASH1 can not only inhibit endothelial cell tubulogenesis activity but also enhance the stress ability of cells. After low expression of VASH1, cells are
easy to be killed by external stimuli. It is suspected that the expression of SIRT1 and SOD2 is activated by VASH1. Miyashita et al. [8] also confirmed this result by interfering with the expression of VASH1 with lentivirus and found that endothelial cells would show autophagy and premature senescence after VASH1 knockdown, and endothelial cells were very easy to die due to external stimulation. Therefore, VASH1 regulates the activity of tumor cells and endothelial cells through a variety of signaling pathways in different tissues.

In the nervous system, Vincent et al. [32] demonstrated that binding VASH1 to SVBP could specifically inhibit tubulin tyrosine/phenylalanine carboxypeptidase activity, thus further promoting glial cell differentiation and migration. In addition, VASH1-SVBP was initially identified as a secreted protein that regulates angiogenesis, acting together to inhibit tumor angiogenesis. In our study, through differential gene and protein PPI maps, we found that VASH1 expression in LGG is closely related to SVBP and the α-tubulin family, and VASH1 mutation specifically leads to abnormal tyrosine and de-tyrosine dynamic cycles of α-tubulin. Thus, it is closely related to cell transformation and tumor invasion. Meanwhile, GSEA enrichment analysis showed that VASH1 was closely related to cell cycle, P53, Notch, and TGF-β signaling pathways. P53 signaling pathway plays an important role in many biological processes that regulate a variety of gene expression, including apoptosis, growth inhibition, inhibiting cell cycle progression, differentiation and accelerate DNA repair, genome instability, and cell stress after aging, it had been hit by methylation, phosphorylation, acetylation, ubiquitin modification after translation, such as control. Wu [33] et al showed that VASH1 was strongly correlated with P53 and TAp53, and inhibited angiogenesis in NSCLC. It is noteworthy that, although some studies have shown that VASH1 mainly plays a role in regulating VEGF through the angiogenesis pathway in esophageal cancer, prostate cancer, and small cell lung cancer. Zhou [34] et al showed that in cervical cancer, VASH1 is regulated by miR-221-3p to activate the ERK-Akt pathway, and has nothing to do with VEGF. Similarly, no correlation between VASH1 and VEGA was found in LGG in this study, which requires more studies to further confirm its molecular mechanism.

In order to further study the specific biological role of VASH1 in LGG, we detected the expression of VASH1 in several human glioma cell lines commonly used in the laboratory and screened them according to the results, and then established the U-251 cell line with VASH1 knockdown. By Transwell invasion assay and scratch healing assay, we found that knocking down VASH1 significantly improved the migration and invasion ability of U-251 cells. It is concluded that VASH1 can inhibit the progression of glioma under certain conditions. According to Zhao [35] et al., VASH1 overexpression can inhibit the proliferation and apoptosis of human umbilical cord endothelial cells and 786-0 cells, but it cannot inhibit tumor invasion. We believe that this may be due to different internal microenvironments of different tumor tissues, resulting in changes in the interaction of cytokines such as VASH1. Finally, the biological function of VASH1 is different. To sum up, we found that the progress VASH1 expression can promote tumor cell lines, instead of in-person LGG in the patient's body, when the tumor mass in the rapid growth stage, through the feedback mechanism produces lots of VASH1 secretion, thus a further reflection of tumor progression and prognosis of patients, which can be used as independent risk factors for the prognosis of patients with LGG. However, since the changes after VASH1 overexpression and related
molecular pathways were not verified in this study, more in vivo studies are needed to further verify these findings. In addition, more detailed mechanisms of VASH1 in LGG genesis and development must be further explored.

**Conclusion**

Based on the above results, we found for the first time that VASH1 was highly expressed in LGG, and the high expression of VASH1 was closely related to the poor prognosis of LGG patients. Meanwhile, bioinformatics and experiments confirmed the high specificity and biological characteristics of VASH1 in LGG and further found that VASH1 may regulate tumor progression through immune-related signaling pathways, P53 signaling pathways, and SVBP/α-tubulin. In conclusion, this study preliminarily suggests that VASH1 can be used as an important prognostic biomarker and potential therapeutic target for LGG, and provides a new molecular layer for screening high-risk LGG.

**Declarations**

**Acknowledgments**

Not applicable

**Authors’ contributions**

The project design was completed by Yirizhati Aili. The operation experiment was carried out by Aierpati maimaiti and Nuersimanguli Maimaitiming. Data analysis for Hu Qin, Wenyu Ji, and Yusufu Mahemuti. Wen Liu finished writing the manuscript. Data review and article review by Yongxin Wang, and Zengliang Wang. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article and its additional files.

**Ethics approval and consent to participate**

All the experimental procedures were approved and executed following the first affiliated hospital of Xinjiang Medical University.

**Consent for publication**

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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**Tables**

**Table. 1** Tumor name and abbreviations.
| Abbreviations | Tumor name |
|---------------|------------|
| ACC           | Adrenocortical Carcinoma |
| BLCA          | Bladder Urothelial Carcinoma |
| BRCA          | Breast invasive carcinoma |
| CESC          | Cervical squamous cell carcinoma and endocervical adenocarcinoma |
| CHOL          | Cholangiocarcinoma |
| COAD          | Colon adenocarcinoma |
| DLBC          | Lymphoid Neoplasm Diffuse Large B-cell Lymphoma |
| ESCA          | Esophageal carcinoma |
| HNSC          | Head and Neck squamous cell carcinoma |
| KICH          | Kidney Chromophobe |
| KIRC          | Kidney renal clear cell carcinoma |
| KIRP          | Kidney renal papillary cell carcinoma |
| LAML          | Acute Myeloid Leukemia |
| LGG           | Lower Grade Glioma |
| LIHC          | Liver hepatocellular carcinoma |
| LUAD          | Lung adenocarcinoma |
| LUSC          | Lung squamous cell carcinoma |
| MESO          | Mesothelioma |
| OV            | Ovarian serous cystadenocarcinoma |
| PAAD          | Pancreatic adenocarcinoma |
| PCPG          | Pheochromocytoma and Paraganglioma |
| PRAD          | Prostate adenocarcinoma |
| READ          | Rectum adenocarcinoma |
| SARC          | Sarcoma |
| SKCM          | Skin Cutaneous Melanoma |
| STAD          | Stomach adenocarcinoma |
| TGCT          | Testicular Germ Cell Tumors |
Table. 2 Correlations between VASH1 expression and clinicopathological characteristics in LGG patients

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| THCA         | Thyroid carcinoma                               |
| UCEC         | Uterine Corpus Endometrial Carcinoma             |
| UCS          | Uterine Carcinosarcoma                           |
| UVM          | Uveal Melanoma                                   |
| Parameters                | VASH1 expression |   | P-value |
|---------------------------|------------------|---|---------|
|                           | High expression(n=57) | Low expression(n=37) | t/χ² |         |
| Age (year)                | 51.5±15.6        | 48.5±16.3       | 0.869 | 0.387   |
| Gender                    | 0.401            | 0.527           |       |         |
| Male                      | 27  28.7%        | 20  21.3%       |       |         |
| Female                    | 30  31.9%        | 17  18.1%       |       |         |
| Histology                 | 2.064            | 0.356           |       |         |
| Astrocytomas              | 39  41.5%        | 27  28.7%       |       |         |
| Oligodendrogliomas        | 5   5.3%         | 4   4.2%        |       |         |
| Ependymomas               | 13  13.8%        | 6   6.5%        |       |         |
| Relapse                   | 17.692           | 0.000           |       |         |
| Primary                   | 21  22.3%        | 30  31.9%       |       |         |
| Recurrence                | 36  38.3%        | 7   7.4%        |       |         |
| Tumor diameter            | 0.296            | 0.586           |       |         |
| <4cm                      | 26  27.7%        | 19  20.2%       |       |         |
| ≥4cm                      | 31  34.4%        | 18  19.1%       |       |         |
| Tumor location            | 1.324            | 0.516           |       |         |
| Frontal lobe              | 20  21.3%        | 16  17.0%       |       |         |
| Temporal lobe             | 24  25.5%        | 20  21.3%       |       |         |
| Other                     | 10  10.6%        | 4   4.3%        |       |         |
| WHO Stage                 | 39.070           | 0.000           |       |         |
| I                         | 1   1.1%         | 11  11.7%       |       |         |
| II                        | 14  14.9%        | 38  40.4%       |       |         |
| III                       | 28  29.8%        | 3   3.33%       |       |         |
| Karnofsky (KPS)           | 3.132            | 0.077           |       |         |
| <70                       | 16  12.7%        | 10  3.33%       |       |         |
| ≥80                       | 28  34.0%        | 40  50.0%       |       |         |
| Epilepsy                  | 12.163           | 0.001           |       |         |
| Yes                       | 29              | 14              |       |         |
| No | 16 | 35 |
|----|----|----|
| IDH1 type | 4.785 | 0.029 |
| Wild | 30 | 11 |
| Mutant | 27 | 26 |

**Table 3** Univariate and multivariate Cox analyses of OS in GBM patients

|                  | Univariate analysis | Multivariate analysis |
|------------------|--------------------|----------------------|
|                  | HR     | 95% CI  | P   | HR     | 95% CI  | P   |
| Age              | 1.01   | 0.99–1.02 | 0.330 | 0.99   | 0.98–1.01 | 0.440 |
| Epilepsy         | 0.93   | 0.62–1.38 | 0.710 | 1.19   | 0.78–1.82 | 0.420 |
| Relapse          | 3.70   | 2.46–5.57 | 0.001 | 3.47   | 2.19–5.48 | 0.000 |
| IDH1 type        | 0.82   | 0.57–1.20 | 0.310 | 1.36   | 0.88–2.12 | 0.170 |
| WHO stage        | 2.81   | 2.23–3.53 | 0.001 | 3.13   | 2.39–4.09 | 0.000 |
| Expression of VASH1 | 1.54  | 1.04–2.26 | 0.030 | 1.65   | 1.08–2.50 | 0.020 |

**Figures**
Figure 1

Abnormal expression of VASH1 in generalized carcinoma. (A) Expression differences of VASH1 in 27 cancer types integrated from GTEx and TCGA databases. (B) VASH1 expression levels in 21 tumor cells from the CCLE database. (C) Forest diagram of the relationship between VASH1 expression and OS in 33 tumors. (D) Forest diagram of the relationship between VASH1 expression and DSS in 33 cancers. (E~G) Kaplan-Meier OS curves of VASH1 expression in KIRC, LGG, LIHC, and PAAD. (H~K) Kaplan-Meier DSS curves of VASH1 expression in KIRC, LGG, LIHC, and PAAD. (*P<0.05, **P<0.01, ***P<0.001)
Figure 2

Correlation analysis between VASH1 expression and immune cell infiltration, matrix score, immune checkpoint genes, and TMB of KIRC, LGG, PAAD, and LIHC cancers that are closely related. (A) VASH1 expression was positively correlated with immune cell infiltration in KIRC, LGG, PAAD, and LIHC. (B) Correlation between VASH1 and ImmuneScore, ESTIMATEScore, and StromalScore in KIRC, LGG, PAAD, and LIHC. (C) VASH1 expression was positively correlated with KIRC, LGG, and LIHC matrix scores. (D) Correlation analysis between VASH1 expression and 47 immune checkpoint genes in cancer. (E)
Correlation analysis between VASH1 expression and TMB in generalized carcinoma. (*P < 0.05, **P < 0.01, ***P < 0.001)

Figure 3

Expression difference of VASH1 gene and somatic variation. (A) VASH1 expression was significantly different in GBM-LGG, LGG, KIPAN, MESO, and SKCM samples. (B) Scatter plot of VASH1 expression related to TMB (P=0.0035). (C) Box diagram of different expressions of VASH1 and TMB (P=0.008). (D)
Kaplan-Meier curves for LGG patients (TCGA-LGG, P<0.001, stratified patients using TMB mutation load and VASH1 expression). (E, F) are respectively oncoPrint constructed based on VASH1 expression level.

**Figure 4**

Survival analysis of LGG patients with VASH1 expression level. (A) Comparison of VASH1 expression with survival time and survival status. (B, C) Difference of VASH1 expression between normal and LGG tissues in GPEIA online database. (D) Kaplan-Meier curves of LGG patients from GPEIA online database. (E) Kaplan-Meier curves of LGG patients from UALCAN online database. (F, H) ROC curves (LGG RNA-seq
samples in TCGA) were performed for correlation between clinical prognostic factors and VASH1 expression levels at different time points in 510 LGG patients.

Figure 5

VASH1 gene characteristics and functional analysis. (A) GO enrichment analysis of VASH1 characteristic gene sets. (B) KEGG enrichment analysis of VASH1-related signaling pathways. (C) String construction of
VASH1 protein co-expression network. (D) GeneMANIA constructed a co-expression network of the VASH1 gene. (E) GSEA analyzed the correlation between VASH1 and the signaling pathway in the KEGG database.

**Figure 6**

Evaluation of the performance of the VASH1 using two external independent CGGA mRNA-seq-693 and CGGA mRNA-seq-325 datasets. (A) Box plots for gene expression levels of VASH1 for patients of a different gender from the CGGA mRNA-seq-693 set. (B) Box plots for gene expression levels of VASH1 for patients at tumor type from the CGGA mRNA-seq-693 set. (C) Box plots for gene expression levels of VASH1 for patients at different WHO grades from the CGGA mRNA-seq-325 set. (D) Survival analysis for VASH1 risk stratification using CGGA mRNA-Seq-693 set. (E) Survival analysis for VASH1 risk stratification using CGGA mRNA-seq-325 set.
Figure 7

Validation of the elevated expression and prognostic value of VASH1. (A) Representative immunohistochemistry staining of VASH1 in LGG, GBM samples, and adjacent normal tissues. (B) Quantified data of the score for VASH1 staining. (C,D) qRT-PCR was used to confirm the expression level of LGG and adjacent Normal tissues. (E) OS curves of patients in the LGG stratified by VASH1 expression levels (Our cohort). (F) DFS curves of patients in the LGG stratified by VASH1 expression levels (Our cohort).
Figure 8

LGG prognostic analysis. (A) value, risk factor HR, and confidence interval for univariate analysis of age, epilepsy, relapse, IDH1, stage, and VASH1 expression. (B) value, risk factor HR, and confidence interval for multivariable analysis of age, epilepsy, relapse, IDH1, stage, and VASH1 expression. (C) a Nomogram diagram of the LGG prognostic prediction model. (D) calibration curve of the column line plot. (E) The ROC curve was used to determine the correlation between the 3-year survival of LGG patients and VASH1 expression level (AUC=0.82). (**P<0.01, ***P<0.001)
Figure 9

The function of VASH1 was confirmed by in vitro experiments. (A ~ D) The expression levels of VASH1 in different cell lines were detected by qRT-PCR and Western Blot, and the highest expression level was found in the U-251 cell line. (E, F) Western Blot detection of interference efficiency of VASH1 in U-251 cells. (F) Detection of interference efficiency of VASH1 in U-251 cells by qRT-PCR. (H, I) Wound-Healing assay indicated that interference of VASH1 expression could promote the migration of U-251 cells. (J, K)
Transwell migration assay showed that interference with VASH1 expression promoted the migration ability of U-251 cells. (*P<0.05, **P<0.01, ***P<0.001)

**Supplementary Files**

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- SupplementaryFig.S1.tif