VAV3 regulates glioblastoma cell proliferation, migration, invasion and cancer stem-like cell self-renewal

RUI MIAO[1], DONG HUANG[1], KAITAO ZHAO[1], YANG LI[1], XIAOMEI ZHANG[1], YI CHENG[1] and NA GUO[2]

[1]Department of Neurology, Shaanxi Second Provincial People's Hospital, Xi'an, Shaanxi 710005;
[2]Department of Immunology, Xi'an Medical University, Xi'an, Shaanxi 710021, P.R. China

Received October 8, 2022; Accepted March 3, 2023

DOI: 10.3892/mmr.2023.12981

Abstract. Glioblastoma multiforme (GBM; World Health Organization grade IV) is one of the most common and aggressive malignant brain tumors and has no effective treatment. Therefore, elucidation of the molecular mechanism of glioma development is very important for finding new therapeutic strategies. The present study evaluated the expression level of Vav guanine nucleotide exchange factor 3 (VAV3) using bioinformatics analysis and demonstrated that VAV3 was overexpressed in human glioblastoma and associated with patient survival. Knock down of VAV3 using shRNA in glioblastoma cells significantly inhibited glioblastoma cell migration, invasion and proliferation. Furthermore, downregulation of VAV3 expression inhibited the stem cell self-renewal capacity and decreased the expression levels of the stem cell markers Nestin and Sox2. Bioinformatic analysis demonstrated that VAV3 was a target gene of miR-218. Furthermore, overexpression of VAV3 markedly reversed the tumor suppressor effect of miR-218 in glioblastoma cell. These findings suggested that VAV3 could be a potential biomarker and therapeutic target for glioblastoma.

Introduction

Glioblastoma (GBM; World Health Organization grade IV) is the most common malignant primary cancer of the central nervous system, and is characterized by aggressive invasiveness and proliferation (1). The incidence of glioblastoma ranges from 0.59 to 5 per 100,000 persons and keeps rising in certain countries, such as Korea and England (2), possibly as a consequence of an aging population, increasing ease of access to neuroimaging, exposure to ionizing radiation, radiofrequency electromagnetic fields and air pollution (3-6). Although progress has been made in diagnostic and prognostic stratification, the 5-year survival of GBM patients is ~5% after initial diagnosis (7) and the median survival is ~14.6 months (8,9). Therefore, elucidation of the molecular mechanism of GBM development is very important for finding new therapeutic strategies.

Vav guanine nucleotide exchange factor 3 (VAV3) is a member of the VAV gene family. The VAV proteins are guanine nucleotide exchange factors (GEFs) which regulate the Rho family GTPases. There are three members of the Vav kinase family: VAV1, VAV2 and VAV3. VAV1 is mainly expressed in hematopoietic cells, whereas VAV2 and VAV3 are ubiquitous in numerous cell types (10,11). VAV family members are highly homologous at the protein level (50-70%) and share an array of structural motifs, therefore they have the overlapping and unique functions (12). Previous studies have reported that VAV3 is an oncogene and regulates numerous cellular signaling process, such as cell proliferation and apoptosis, gene transcription, and cytoskeleton organization (13,14). VAV3 has been reported to be overexpressed in numerous human cancers, including prostate cancer, endometrial cancer, breast cancer and colorectal cancer (10,14-16). VAV3 can regulate prostate cancer cell growth via activation of the androgen receptor-mediated signaling axis (15). VAV3 overexpression is directly correlated with decreased survival in gastric cancer, and knockdown of VAV3 has been reported to inhibit cancer proliferation both *in vitro* and *in vivo* (17). A study reported that the Rac-activating GEFs Trio, Ect2 and Vav3 regulated glioblastoma cell migration and invasion (18). However, the expression and prognostic significance of VAV3 in glioblastoma is still unknown and needs to be further assessed.

Materials and methods

Cell lines and cancer stem-like cell culture. U251 cells (cat no. TCHu58) and U87 glioblastoma of unknown origin (cat. no. TCHu138) cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences (Shanghai, China) and the cell lines were identified by STR. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The serum-free medium (SFM) was composed of DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) with 20 μl/ml B27 supplement (Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (MilliporeSigma) and 20 ng/ml epidermal growth factor (MilliporeSigma).

Correspondence to: Dr Na Guo, Department of Immunology, Xi'an Medical University, 1 Xinwang Road, Xi'an, Shaanxi 710021, P.R. China
E-mail: guona1214@hotmail.com

Key words: glioblastoma multiforme, Vav guanine nucleotide exchange factor 3, miR-218, proliferation, stemness

Glioblastoma stem-like cells (GSCs) were isolated from U251 cells transfected with shCTRL or shVAV3-#1/2 using the serum-free medium (SFM). These cells can form neurosphere-like cell aggregates in <7 days (19). For the selection of stable glioma cell lines, U251 cells were cultured in 400 μg/ml neomycin for 14 days after transfection.

RT-qPCR. mRNA expression of VAV3 in human glioblastoma samples was analyzed by the online database GlioVis (gliovis.bioinfo.cnio.es/). Total RNA from cells was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The total RNA was subsequently treated with RNase-free DNase I (Roche Diagnostics). Complementary DNA synthesis was performed using a BcaBest RNA PCR kit from Takara Bio, Inc. according to the manufacturer's protocols. qPCR was performed using an iQ™5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) with Realtime PCR Master Mix (SYBR Green, Toyobo Life Science). The thermocycling conditions used for qPCR were as follows: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, in a total volume of 20 μl. Relative mRNA expression levels were assessed using the $2^{-\Delta\Delta Cq}$ method (20). GAPDH was used as the endogenous control. The PCR primer sequences used were as follows: VAV3 forward (F): 5'-CTGCCAGCTGCTTAACAACC-3' and reverse (R), 5'-CAGGCCGTGAGAAATGTCCT-3'; miR-218 RT primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATGG-3' (21); miR-218 qPCR F primer, 5'-GTGCAGGGTCCGAGGTATTC-3', miR-218 qPCR R primer, 5'-TTGATCTAACCATGTGTCGTA-3'; and GAPDH F, 5'-GAAGGTGAAGGTCGGAGTC-3' and R, 5'-GAAGATGGTGATGGGATTTC-3'.

Western blotting. The glioblastoma cells lysates were prepared using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.) with complete protease inhibitor cocktail (Roche Diagnostics). The protein concentration was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The samples (20 μg/line) were separated by 12 % SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche Diagnostics). The membranes were blocked using 5% non-fat dry milk in TBS, followed by incubation with the primary antibodies at 4˚C overnight and then HRP-labeled secondary antibodies at room temperature for an hour (Cat#: 61-6520, 1:5,000, HRP-labeled, Thermo Fisher Scientific, Inc). The immunolabeled proteins were detected using an ECL detection system (Wuhan Boster Biological Technology, Ltd). The primary antibodies used were as follows: Actin (1:1,000, cat. no. AA128; Beyotime Institute of Biotechnology), VAV3 (1:1,000, cat. no. 2398s, Cell Signaling Technology, Inc.), Nestin (1:1,000, cat. no. MAB5326, MilliporeSigma) and SOX2 (1:1,000, cat. no. 3579s, Cell Signaling Technology, Inc.).

VAV3 shRNA synthesis and transfection. Cell transfections with 1 μg nucleic acid were performed using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Inc) according to the manufacturer's protocol. For the selection of stable cell lines, the transformed cells were cultured in 400 ug/ml G418 for 14 days and the expression of VAV3 was assessed using western blotting and RT-qPCR. The sequences of the shRNAs were as follows: VAV3 shRNA-1#, CGAAGTTGTTGTCTAGCAGAA and VAV3 shRNA-2#, CGGAACCTAATGCAAGAGATT.

Luciferase assay. The binding site of miR-218 to VAV3 was predicted by the online database Target scan (targetscan.org/vert_80/; version 8.0). The miR-218 vector and pmirGLO-VAV3-3'UTR, pmirGLO-VAV3-3'UTR-mutant (mut), were co-transfected into U251 cells using Lipofectamine™ 2000 transfection reagent(Thermo Fisher Scientific, Inc). The cells were harvested and lysed at 24 h post-transfection. The luciferase activity was measured using the dual-luciferase reporter assay system (Promega Corporation) according to the manufacturer's protocol. A total of 48 h post-transfection, firefly and Renilla luciferase activities were detected using the GloMax®-Multi Jr Single Tube Multimode Reader (Promega, Madison, WI, USA), according to the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA).

In vitro migration and invasion assays. Glioblastoma Cells ($5x10^5$) were added on the top side of polycarbonate Transwell filters (without Matrigel for Transwell migration assays) or plated on the top side of polycarbonate Transwell filter coated with Matrigel at 37˚C for an hour (for Transwell invasion assay) in the upper chamber of the QCM™ 24-Well Cell Invasion Assay (Cell Biolabs, Inc.) plates. For Transwell migration assays, cells were suspended in SFM and SFM was used in the lower chamber. For the invasion assay, cells were suspended in SFM and DMEM with fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was used as a chemoattractant in the lower chamber. The cells were incubated at 37˚C for 8 h (migration assay) or 48 h (invasion assay). The cells in the top chambers were then removed using cotton swabs. The migrated and invaded cells on the lower membrane surface were fixed using 100% methanol for 10 min at room temperature, air-dried, then stained with DAPI at room temperature for 10 min and quantified using a fluorescence OLYMPUS U-HGLGPS microscope and cellSens entry software 1.0 (Olympus, Japan).

Wound healing assays. U251 cells were seeded in six-well plates and cultured at 37˚C overnight in DMEM medium until 100% confluency. A wound was then created by manually scraping the cell monolayer with a 200 μl pipette tip. The cultures were washed twice with PBS to remove floating cells. The cells were then incubated at 37˚C in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cell migration into the wound was assessed at three preselected time points (0, 24 and 48 h) in eight randomly selected fields of view for each condition and time point. Images were captured using a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope. The distance traveled by the cells was determined by measuring the wound width at different time points and then subtracting it from the wound width at 0 h using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD).

Limiting dilution assay. A limiting dilution assay was performed as described previously (19,22). Cancer sphere cells

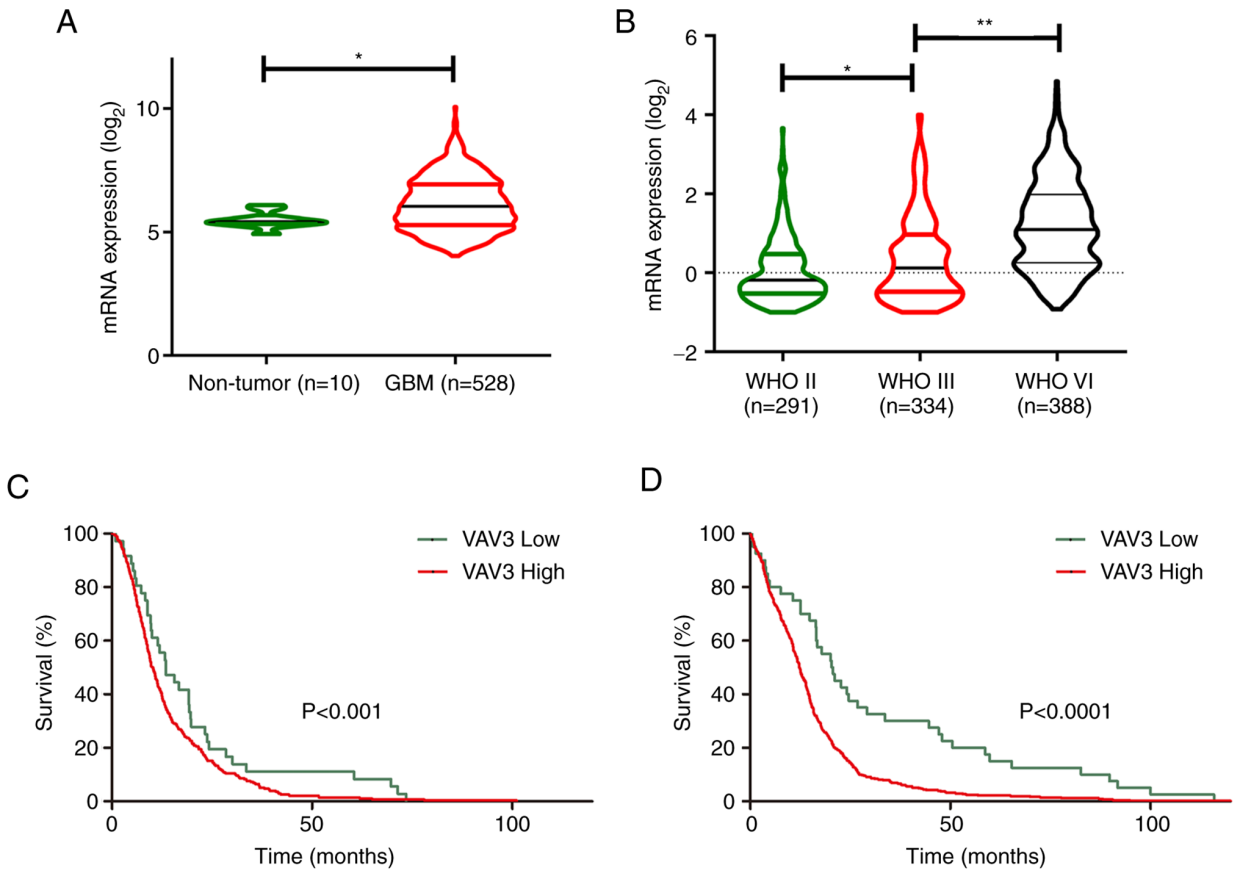

Figure 1. VAV3 expression in human glioblastoma. (A) VAV3 mRNA expression levels in human glioblastoma and normal tissues from TCGA database. (B) VAV3 mRNA expression levels in human glioblastoma in the CGGA database. Kaplan-Meier survival analysis demonstrated that VAV3 expression was negatively correlated with patient survival in data from the (C) TCGA and (D) CGGA databases. *P<0.05 and **P<0.01. VAV3, Vav guanine nucleotide exchange factor 3; GBM, glioblastoma multiforme; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas; WHO, World Health Organization.

were dissociated and plated on 96-well micro-well plates in 0.2 ml SFM. Final cell dilutions ranged from 20 cells/well to 160 cell/well in a total volume of 0.2 ml. Cultures were added with 0.025 ml of SFM every 2 days until day 7, then the percentage of wells without spheres was calculated for each cell plating density and plotted against the number of cells per well. Limiting dilution analysis was performed using online software ELDA 1.0 (Extreme Limiting Dilution Analysis) (http://bioinf.wehi.edu.au/software/elda/).

Cell counting kit-8 (CCK8). U251 cells (3000 cells/well in 100 μl DMEM medium) were seeded into 96 well tissue culture plate, each group was repeated 3 times. CCK8 (Selleck Chemicals) regent was add with 10 μl/well at four time points (24, 48, 72 and 96 h), incubated 2 h at 37˚C; after shaking the plates for 1 min, the absorbance of samples was measured using an ELISA reader at 450 nm.

Animal studies. The present study was compliant with all relevant ethical regulations regarding animal research and was approved by the Institutional Animal Care and Use Committee of Xi'an Medical University (No. XYLS2022190). U251 cells stably expressing VAV3-shRNA-1# or empty vector controls ($4.0x10^6$ cells in 200 μl per mouse) were injected subcutaneously in the flanks of female athymic nude BALB/c nu/nu mice (n=6 per treatment group; age, ~6 weeks). Mice were housed in a standard environment which was characterized by 12 h light/dark cycle, 22-25˚C and 40-60% humidity with free access to water and chow. Tumor volumes were calculated according to the formula: Volume=length x $width^2/2$. The experiment lasted for 20 days and no animals died during the experimental period. Animal health was monitored every day and the size of the tumor was measured every four days. A total of six mice per group were used and tumor cells were injected in both sides to minimize the number of mice used. The mice were euthanized when a single tumor volume reached 3,000 mm^3. The mice were euthanized cervical dislocation and death was confirmed when mice stopped breathing and had no nerve reflex.

Statistical analysis. In vitro experiments were performed with at least three independent biological samples unless otherwise stated. Data were presented as mean ± standard error. Comparisons between two groups were performed using the Student's t-test. Comparisons among multiple groups were performed using one-way ANOVA followed by Dunnett's post-hoc test. Survival of animals in *in vivo* studies was analyzed using Kaplan-Meier analyses and log-rank test. GraphPad Prism 8.0 for windows (GraphPad Software, Inc.) was used for data analysis. P<0.05 was considered to indicate a statistically significant difference.

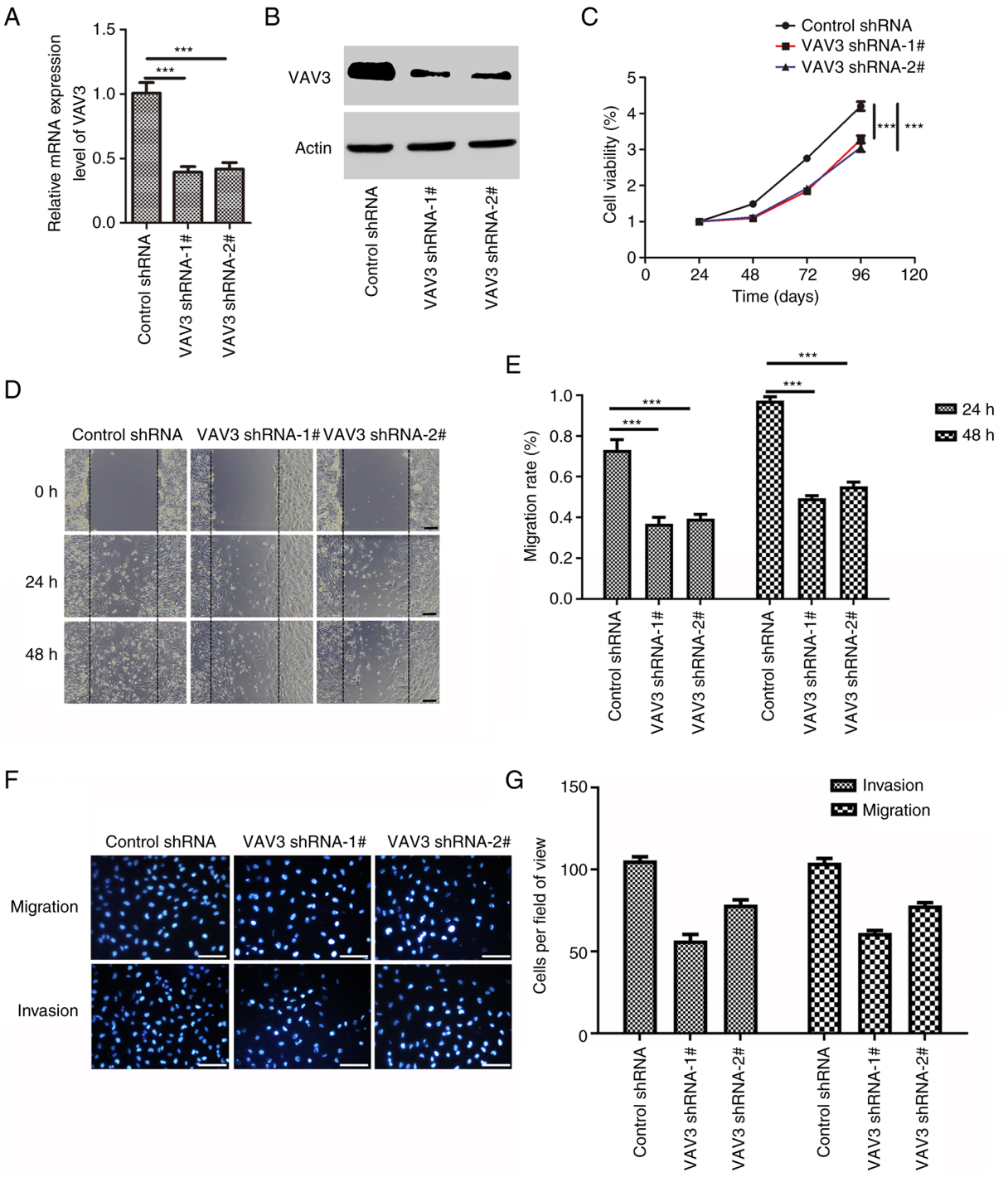

Figure 2. Reduced VAV3 inhibits U251 glioblastoma cell proliferation *in vitro*. (A) Reverse transcription-quantitative PCR analysis demonstrated theVAV3 mRNA expression level in U251 cells with endogenous VAV3 suppressed by shRNAs. (B) Western blotting analysis of VAV3 protein expression levels in U251 cells with endogenous VAV3 suppressed with shRNAs. (C) Knockdown of VAV3 inhibited cell proliferation in U251 cells. The wound healing assay demonstrated different cell migration rates in control shRNA-U251, VAV3 shRNA-1#-U251 and VAV3 shRNA-2#-U251 cells. (D) Representative images were taken at different time points. (E) Quantification of cell motility was assessed by measuring the wound width. Transwell assays of U251 cells transfected with control shRNA, VAV3 shRNA-1# and VAV3 shRNA-2#. (F) Representative fields of view of invasive cells and migratory cells. (G) Quantitative analysis of the invasive and migratory cells from three independent experiments. Scale bar=50 μm. Error bars represented standard error. ***P<0.001. VAV3, Vav guanine nucleotide exchange factor 3; shRNA, short hairpin RNA.

Results

VAV3 oncogene is overexpressed in human glioblastoma and associated with patient survival. To evaluate whether VAV3 was crucial for human glioblastoma tumorigenesis, the publicly available data at GlioVis (23) were assessed. Analysis of GlioVis by The Cancer Genome Atlas (TCGA) demonstrated that VAV3 mRNA was expressed at a significantly higher level in GBM compared with normal brain tissues (Fig. 1A). Further analysis of the Chinese Glioma Genome Atlas (CGGA) (24), demonstrated that the mRNA expression level of VAV3 was significantly increased with increasing tumor grade (Fig. 1B),

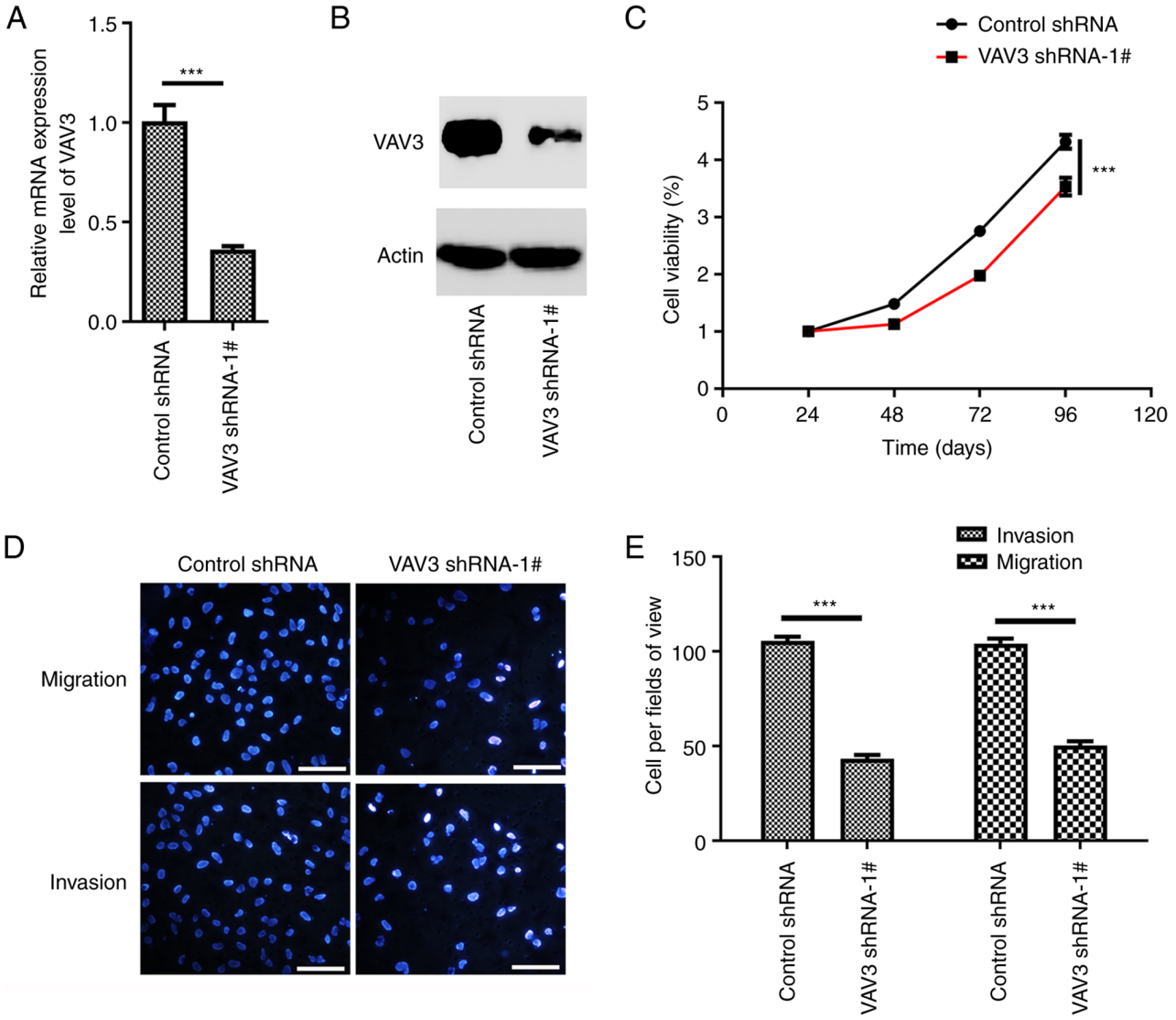

Figure 3. Decreased VAV3 inhibited U87 glioblastoma cell proliferation *in vitro*. (A) Reverse transcription-quantitative PCR analysis demonstrated that VAV3 expression in U87 cells with endogenous VAV3 was suppressed by shRNAs. (B) Western blotting analysis of VAV3 protein expression levels in U87 cells with endogenous VAV3 suppressed by shRNAs. (C) Knockdown of VAV3 inhibited cell proliferation in U87 cells. (D) Representative fields of view of invasive cells and migratory cells. (E) Quantitative analysis of the invasive and migratory cells from three independent experiments. Scale bar=50 μm. Error bars represented standard error. ***P<0.001. VAV3, Vav guanine nucleotide exchange factor 3; shRNA, short hairpin RNA.

the levels of VAV3 expression in tumor specimens were associated with their World Health Organization grades. Kaplan-Meier analysis using the optimal cutoff demonstrated that mRNA expression levels of VAV3 in GBM specimens were inversely associated with patients' survival time based on data from TCGA (Fig. 1C) and CGGA (Fig. 1D). Collectively, these analyses suggested that VAV3 was an oncogene and could be a potential therapeutic target for GBM therapy.

VAV3 knockdown inhibits U251 glioblastoma cell migration, invasion and proliferation. To evaluate the function of VAV3 in glioblastoma cells, shRNA targeting VAV3 mRNA (shVAV3) was used to suppress the expression of VAV3 in U251 cells. The knockdown effect of shRNA was assessed using RT-qPCR and western blotting analysis (Fig. 2A and B). The results demonstrated that both the VAV3 shRNAs markedly suppressed VAV3 mRNA and protein expression levels and that VAV3-shRNA-1# was better. CCK-8 cell viability assays demonstrated that the suppression of the expression of VAV3 in U251 cells significantly inhibited the proliferation of glioblastoma cells compared with the control (Fig. 2C). The wound healing assay demonstrated that reduced VAV3 expression significantly suppressed the migration ability of U251 cells compared with the control (Fig. 2D and E). Furthermore, the migration and invasive potential of VAV3 shRNA U251 cells was also markedly reduced compared with the control (Fig. 2F and G). All these suggest that VAV3 regulate the U251 glioblastoma cell migration, invasion and proliferation, migration and invasion.

VAV3 knockdown inhibits U87 migration, invasion and proliferation. To evaluate the role of VAV3 in glioblastoma cells, VAV3-shRNA-1# was used to specifically suppress the expression of VAV3 in U87 cells. The knockdown effect of shRNA was assessed using RT-qPCR and western blotting analysis (Fig. 3A and B). The results demonstrated that the VAV3 shRNA could markedly suppress VAV3 expression in

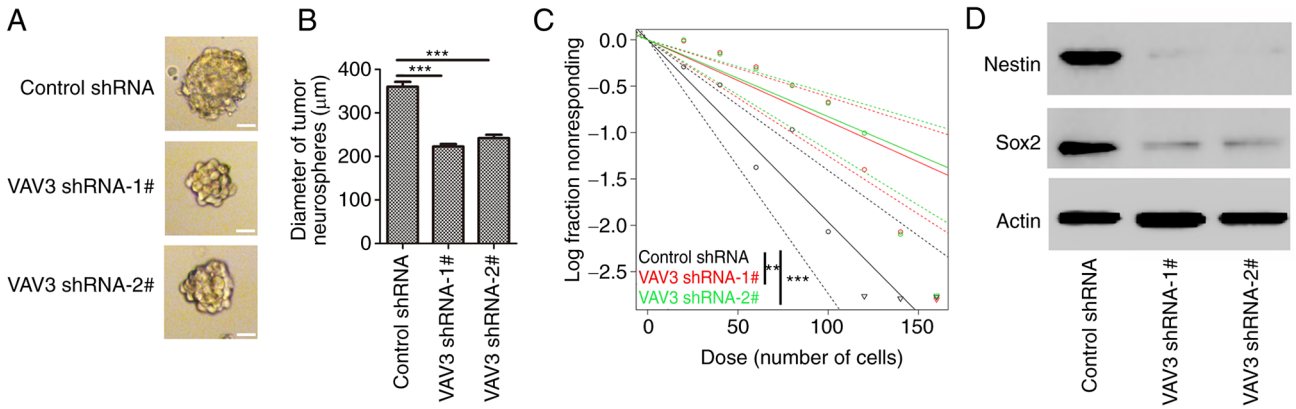

Figure 4. Targeting VAV3 with shRNA decreased glioblastoma stem-like cells self-renewal. (A) The morphology of tumor spheres formed by the cancer stem cells from control shRNA U251 cells, VAV3 shRNA-1#-U251 cells and VAV3 shRNA-2#-U251 cells. (B) Glioblastoma tumor sphere diameters decreased in VAV3-shRNAs U251 GBM stemlike cells. (C) *In vitro* extreme limiting dilution assays to single cells demonstrated that knockdown of VAV3 in U251 cells decreased the frequency of tumor sphere formation (D) Western blotting analysis demonstrated the protein expression levels of Nestin and Sox2 in neurospheres derived from control shRNA U251, VAV3 shRNA-1#-U251 and VAV3 shRNA-2#-U251 cells. Scale bar=100 μm. Error bars represented standard error. **P<0.05. ***P<0.001. VAV3, Vav guanine nucleotide exchange factor 3; shRNA, short hairpin RNA.

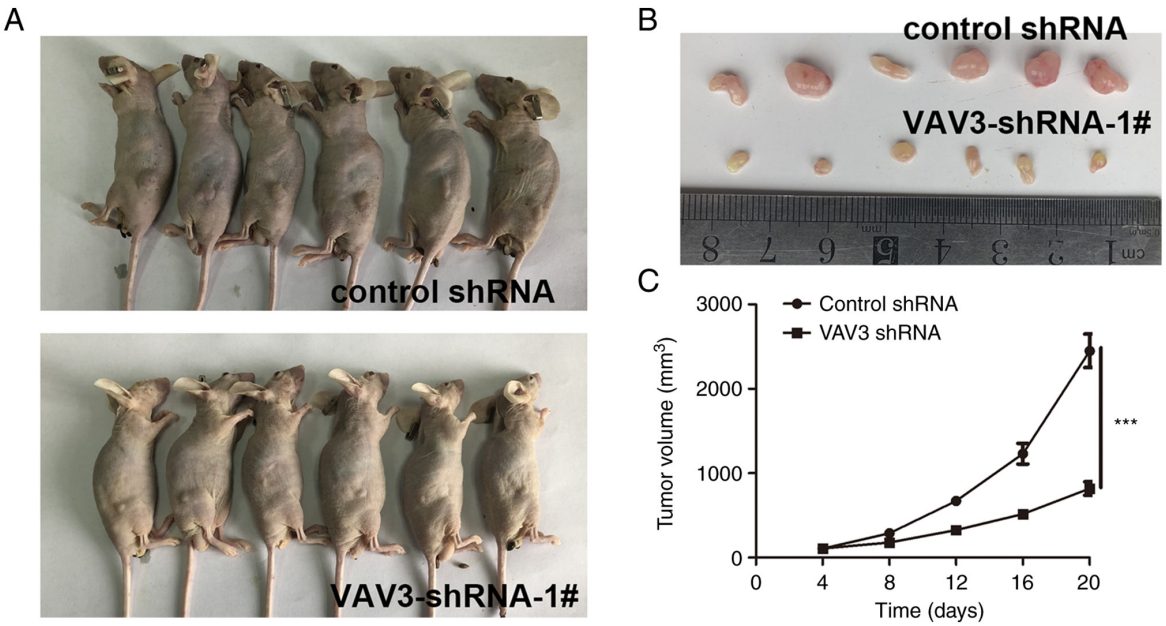

Figure 5. VAV3 shRNAs significantly decreased tumor growth in a xenograft model. (A) Photographs of tumorigenesis in U251 cells in xenograft mice. (B) Photographs of the isolated tumor tissues. (C) The volume of the xenograft tumors were measured at the indicated time points. Data are expressed as mean ± standard deviation (n=6). ***P<0.001. VAV3, Vav guanine nucleotide exchange factor 3; shRNA, short hairpin RNA.

U87 cells compared with the control. CCK-8 cell viability assays demonstrated that the suppression of the expression of VAV3 in U87 cells significantly inhibited the proliferation of glioblastoma cells compared with the control (Fig. 3C). Furthermore, the migration and invasion ability of VAV3 shRNA U87 cells was also significantly reduced compared with the control (Fig. 3D and E). Collectively, these analyses suggested that VAV3 regulate the U87 glioblastoma cell migration, invasion and proliferation, migration and invasion.

VAV3 knockdown inhibits the self-renewal capacity of glioblastoma stem-like cells. The effects of VAV3 on glioblastoma stem like cell self-renewal, U251 cells were cultured in SFM to induce the tumor sphere formation. A significant decrease in the diameter of U251 neurospheres in VAV3 knockdown groups (VAV3 shRNA-1#, 217.3±9.9 μm; VAV3 shRNA-2#, 235.2±14.8 μm) was demonstrated compared with that of the control (311.5±15.4 μm) (Fig. 4A and B). Furthermore, down-regulation of VAV3 reduced the self-renewal capacity of U251 cancer stem-like cells compared with the control. The number of cells required to generate at least one tumor sphere/well was 113.9 μm in VAV3 shRNA-1#-U251 cells, 120.6 μm in VAV3 shRNA-2#-U251 cells, and 50.4 μm in control shRNA-U251 cell (Fig. 4C). The protein expression levels of the stem cell markers Nestin and Sox2 in these tumor neurospheres was assessed using western blotting, which demonstrated that the expression of these GSC makers were markedly decreased in the tumor spheres from VAV3 shRNA-transfected U251 cells

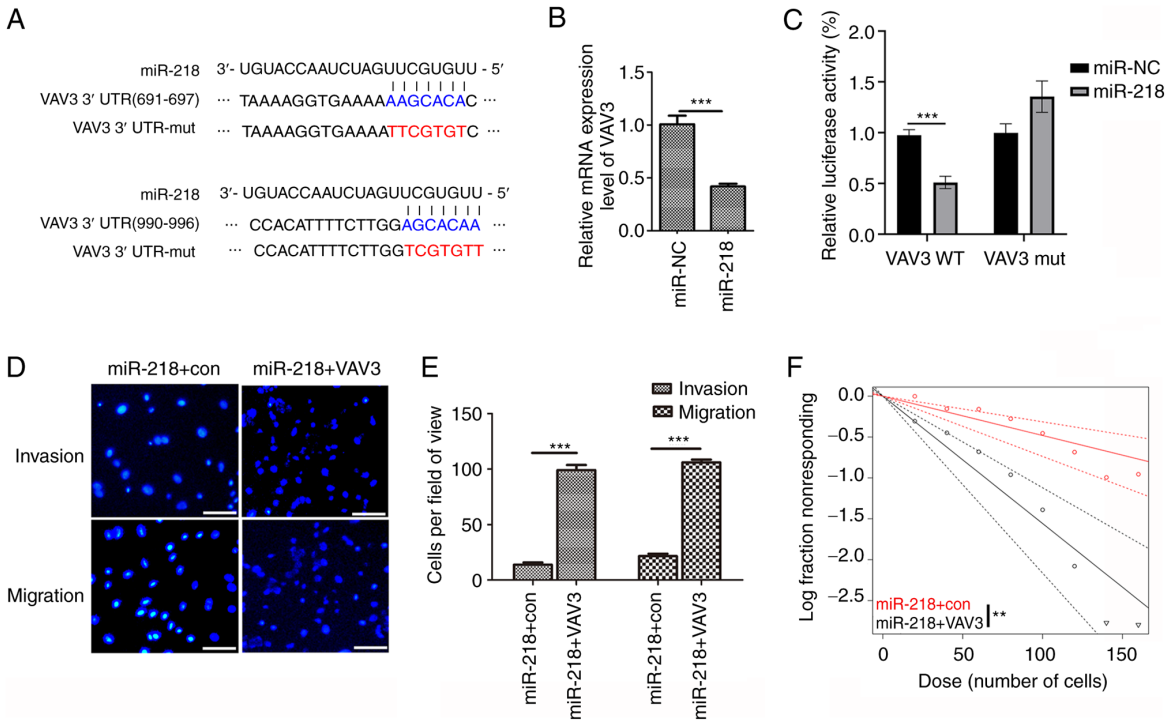

Figure 6. miR-218 targets VAV3 by binding to its 3'UTR. (A) Predicted miR-218 target sequences in the 3'UTR of VAV3 and mut containing eight mutated nucleotides in the 3'UTR of VAV3. (B) Reverse transcription-quantitative PCR analysis of VAV3 mRNA expression levels in glioblastoma cells transfected with miR-218 or negative control. (C) U251 cells were co-transfected with miR-218 and luciferase reporters containing either the predicted miRNA target site in VAV3 3'UTR or its corresponding MUT form. (D) Quantitative analysis of the invasive and migratory cells from three independent experiments. (E) Extreme limiting dilution assays to single cells demonstrated that VAV3 overexpression in U251MG cells increased the frequency of tumor sphere formation. Error bars represented standard error. **P<0.05. ***P<0.001. VAV3, Vav guanine nucleotide exchange factor 3; miR, microRNA; WT, wild-type; mut, mutant; con, control; NC, negative control.

compared with control shRNA (Fig. 4D). These data suggested that the downregulation of VAV3 expression could inhibit the self-renewal capacity of glioblastoma stem-like cells.

VAV3 inhibits the glioblastoma progression in vivo. To analyze the role of VAV3 in glioblastoma carcinogenesis, the effect of VAV3 decrease on tumor growth were assessed *in vivo*. VAV3-shRNA-1#-U251 cells and control cells were implanted into the left and right flanks of nude mice (n=6 per group) by subcutaneous injection. At 20 days post-injection, the tumors derived from VAV3-shRNA-1#-U251 cells were significantly smaller than those derived from control-U251 cells (Fig. 5). Which demonstrated that knockdown of VAV3 significantly inhibited the proliferation of glioblastoma cells *in vivo*.

VAV3 is a target gene of miR-218 and overexpression of VAV3 eliminated the effects of miR-218 on U251 glioblastoma cells. Several computational methods were used to evaluate the potential targets of miR-218 in humans. The TargetScan program identified two conserved binding sites for miR-218 in the 3'UTR region of the VAV3 mRNA) (Fig. 6A). To evaluate if VAV3 was a direct target of miR-218, RT-qPCR was used to assess VAV3 mRNA expression levels in U251 cells, which demonstrated significantly decreased VAV3 mRNA expression levels in miR-218 overexpression cells compared with the control (Figs. 6B and S1). To assess whether miR-218 directly targeted VAV3, a dual-luciferase reporter system containing the wild-type (WT) and mut 3'-UTR of VAV3 was used. The luciferase activity in the miR-218/WT-VAV3-UTR-transfected cells was significantly decreased compared with the control, whereas the luciferase activity in the miR-218/mut-VAV3-UTR-transfected cells demonstrated no significant difference, which suggested that miR-218 directly targeted the 3'-UTR of VAV3. Furthermore, miR-218 and the VAV3 overexpression vector were co-transfected into U251 cells to assess whether miR-218 performed its tumor suppression functions via targeting of VAV3. After co-transfection, it was demonstrated that the overexpression of VAV3 significantly counteracted the tumor suppressor effect of miR-218 in glioblastoma cells during migration and invasion (Fig. 6D). Further analysis demonstrated that VAV3 overexpression markedly reduced the effects of miR-218 on the self-renewal capacity of glioblastoma stem-like cells (Fig. 6E). These findings further demonstrated that VAV3 was a target gene of miR-218.

Discussion

Glioblastoma is a lethal brain cancers and VAV3 was demonstrated to be up-regulated in glioblastoma and inversely associated with patients' survival time through systematic bioinformatic analyses. The results of the present study suggested that VAV3 was an oncogene and may serve an important role in the regulation of glioblastoma tumorigenesis. Moreover, the results of the present study demonstrated that knockdown of VAV3 expression by shRNA markedly suppressed GBM cell migration, invasion, proliferation, and glioblastoma stem-like cells self-renewal. Furthermore, knockdown of VAV3 significantly inhibited the proliferation of two glioblastoma cells

in vitro. These data suggested that VAV3 could be a prognostic marker or therapeutic target in glioblastoma.

VAV3 has been reported to be highly expressed in numerous types of cancer (10,14,25). Salhia *et al* (18) reported that the three guanine nucleotide exchange factors trio (trio Rho guanine nucleotide exchange factor), Ect2 (epithelial cell transforming 2) and Vav3 were expressed at higher levels in GBM compared with low-grade glioma in 2008. This was consistent with the results of the present study, which analyzed the TCGA and CGGA databases in 2022, with the number of patient samples analyzed being much higher than in 2008. Furthermore, the results of the present study demonstrated that knockdown of VAV3 markedly inhibited the migration and invasion of glioblastoma cells. Uen *et al* (14) reported that VAV3 knockdown could regulate the cell cycle and metastasis-related molecules via inhibition of the PI3K-AKT signaling pathway in colorectal cancer cells. Recently, Nayak (26) reported that nuclear VAV3 served an important role in B-cell lymphoblastic leukemogenesis through regulation of nuclear Bmi1 phosphorylation and polycomb repression complex-1 (PRC1) activity. the role of BMI 1 in the regulation of glioblastoma migration, invasion and stem cell renewal has been reported by numerous previous studies (27-30). Therefore, VAV3 could regulate glioblastoma migration, invasion and stem cell stemness via regulation of the PI3K-AKT signaling pathway or PRC1 activity.

VAV3 belongs to the GEF family which regulate the Rho family GTPases. Previous studies have reported that the Rho family small GTPases serve an important role in the regulation of the cytoskeleton dynamics, cell proliferation, invasion and survival of cancer cells (31-33). Salhia *et al* (18) reported that the GEFs factors trio, Ect2 and Vav3 serve an important role in the regulation of glioblastoma invasion. As these GEFs factors act on the small GTPase RhoG, the role of RhoG in the regulation of the invasive behavior of glioblastoma cells has been previously assessed, a previous study reported that GTPase RhoG mediated glioblastoma cell invasion and promoted glioblastoma cell survival (34). The present study also demonstrated that VAV3 regulated glioblastoma cell migration and invasion. These data indicated that VAV3 may mediate downstream effectors such as RhoGases to regulate glioblastoma migration and invasion.

A previous study reported that miRNAs could regulate numerous cancer processes (35). Numerous mRNAs have been reported to serve key roles in glioblastoma cell migration, invasion, proliferation, apoptosis and stem cell stemness (36-39). Because of their critical roles in glioma development, miRNAs have been considered as potential biomarkers and therapeutic targets (40). miR-218 has been reported to be down-regulated in glioblastoma and to have inhibited glioma invasion, migration, proliferation and cancer stem-like cell self-renewal (21). In the present study, miRNA-218 target analysis indicated VAV3 as a new direct target of miR-218. Bioinformatic analysis indicated that miR-218 could bind to the VAV3 3'-UTR at two sites: 691-697 and 990-996 nt. The dual-luciferase reporter and RT-qPCR assay demonstrated that miR-218 could directly target VAV3 by recognizing the 3'-UTR of VAV3 mRNA and significantly reduced VAV3 expression levels. Moreover, overexpression of VAV3 markedly reversed the tumor suppressor effect of miR-218 in glioblastoma cells. These results further demonstrated that VAV3 may be a target gene of miR-218 and could regulate glioblastoma tumorigenesis.

This study has several limitations. First of all, the experiment was only carried out in two glioma cell lines, and more glioma cell lines should be used in future research to verify the experimental results. Second, we only studied the function of VAV3 in vitro. In the future, it is necessary to verify the anti-tumor effect of miR-218 and VAV in clinical samples of glioblastoma.

In conclusion, the results of the present study demonstrated that VAV3 could regulate glioblastoma cell migration, invasion, proliferation and glioblastoma stem-like cell self-renewal. For the first time, to the best of our knowledge, the present study demonstrate that VAV3 is a target gene of miR-218 which regulates glioblastoma development by targeting VAV3, which indicates that VAV3 could be a novel therapeutic target for suppressing GBM invasion and recurrence.

Acknowledgements

Not applicable.

Funding

The present study was supported by The Natural Science Basic Research Plan in Shaanxi Province of China (grant no. 2021SF-273), Scientific Research Program Funded by Shaanxi Provincial Education Department (grant no. 20JG029) and Shaanxi Province Innovation capability support plan project (grant no. 2023-CX-PT-03).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RM and NG performed the experiments and contributed to the writing of the manuscript. DH, KZ, YL, XZ and YC performed experiments and contributed to the writing of the article. RM and NG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Xi'an Medical University (No. XYLS2022190).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Torrisi F, Alberghina C, D'Aprile S, Pavone AM, Longhitano L, Giallongo S, Tibullo D, Di Rosa M, Zappalà A, Cammarata FP, *et al*: The hallmarks of glioblastoma: Heterogeneity, intercellular crosstalk and molecular signature of invasiveness and progression. Biomedicines 10: 806, 2022.

2. Grech N, Dalli T, Mizzi S, Meilak L, Calleja N and Zrinzo A: Rising incidence of glioblastoma multiforme in a well-defined population. Cureus 12: e8195, 2020.
3. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Non-ionizing radiation, Part 2: Radiofrequency electromagnetic fields. IARC Monogr Eval Carcinog Risks Hum 102: 1-460, 2013.
4. Fuks KB, Weinmayr G, Basagana X, Gruzieva O, Hampel R, Oftedal B, Sørensen M, Wolf K, Aamodt G, Aasvang GM, *et al*: Long-term exposure to ambient air pollution and traffic noise and incident hypertension in seven cohorts of the European study of cohorts for air pollution effects (ESCAPE). Eur Heart J 38: 983-990, 2017.
5. Smetana K Jr, Lacina L, Szabo P, Dvorankova B, Broz P and Sedo A: Ageing as an important risk factor for cancer. Anticancer Res 36: 5009-5017, 2016.
6. Trylcova J, Busek P, Smetana K Jr, Balaziova E, Dvorankova B, Mifkova A and Sedo A: Effect of cancer-associated fibroblasts on the migration of glioma cells in vitro. Tumour Biol 36: 5873-5879, 2015.
7. Chang K, Zhang B, Guo X, Zong M, Rahman R, Sanchez D, Winder N, Reardon DA, Zhao B, Wen PY and Huang RY: Multimodal imaging patterns predict survival in recurrent glioblastoma patients treated with bevacizumab. Neuro Oncol 18: 1680-1687, 2016.
8. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJB, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, *et al*: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466, 2009.
9. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New Eng J Med 352: 987-996, 2005.
10. Lee K, Liu Y, Mo JQ, Zhang J, Dong Z and Lu S: Vav3 oncogene activates estrogen receptor and its overexpression may be involved in human breast cancer. BMC Cancer 8: 158, 2008.
11. Rahaman SO, Li W and Silverstein RL: Vav Guanine nucleotide exchange factors regulate atherosclerotic lesion development in mice. Arterioscler Thromb Vasc Biol 33: 2053-2057, 2013.
12. Fujikawa K, Inoue Y, Sakai M, Koyama Y, Nishi S, Funada R, Alt FW and Swat W: Vav3 is regulated during the cell cycle and effects cell division. Proc Natl Acad Sci USA 99: 4313-4318, 2002.
13. Hornstein I, Alcover A and Katzav S: Vav proteins, masters of the world of cytoskeleton organization. Cel Signal 16: 1-11, 2004.
14. Uen YH, Fang CL, Hseu YC, Shen PC, Yang HL, Wen KS, Hung ST, Wang LH and Lin KY: VAV3 oncogene expression in colorectal cancer: Clinical aspects and functional characterization. Sci Rep 5: 9360, 2015.
15. Lyons LS and Burnstein KL: Vav3, a Rho GTPase guanine nucleotide exchange factor, increases during progression to androgen independence in prostate cancer cells and potentiates androgen receptor transcriptional activity. Mol Endocrinol 20: 1061-1072, 2006.
16. Boesch M, Sopper S, Marth C, Fiegl H, Wiedemair A, Rössler J, Hatina J, Wolf D, Reimer D and Zeimet AG: Evaluation of Vav3.1 as prognostic marker in endometrial cancer. J Cancer Res Clin Oncol 144: 2067-2076, 2018.
17. Tan B, Li Y, Zhao Q, Fan L, Wang D and Liu Y: Inhibition of gastric cancer cell growth and invasion through siRNA-mediated knockdown of guanine nucleotide exchange factor Vav3. Tumour Biol 35: 1481-1488, 2014.
18. Salhia B, Tran NL, Chan A, Wolf A, Nakada M, Rutka F, Ennis M, McDonough WS, Berens ME, Symons M and Rutka JT: The guanine nucleotide exchange factors trio, Ect2, and Vav3 mediate the invasive behavior of glioblastoma. Am J Pathol 173: 1828-1838, 2008.
19. Qiang L, Yang Y, Ma YJ, Chen FH, Zhang LB, Liu W, Qi Q, Lu N, Tao L, Wang XT, *et al*: Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. Cancer Lett 279: 13-21, 2009.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Tu Y, Gao X, Li G, Fu H, Cui D, Liu H, Jin W and Zhang Y: MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene Bmi1. Cancer Res 73: 6046-6055, 2013.
22. Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF and van der Kooy D: Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208: 166-188, 1999.
23. Bowman RL, Wang Q, Carro A, Verhaak RG and Squatrito M: GlioVis data portal for visualization and analysis of brain tumor expression datasets. Neuro Oncol 19: 139-141, 2017.
24. Zhao Z, Zhang KN, Wang Q, Li G, Zeng F, Zhang Y, Wu F, Chai R, Wang Z, Zhang C, *et al*: Chinese glioma genome atlas (CGGA): A comprehensive resource with functional genomic data from chinese glioma patients. Genomics Proteomics Bioinformatics 19: 1-12, 2021.
25. Liu Y, Mo JQ, Hu Q, Boivin G, Levin L, Lu S, Yang D, Dong Z and Lu S: Targeted overexpression of vav3 oncogene in prostatic epithelium induces nonbacterial prostatitis and prostate cancer. Cancer Res 68: 6396-6406, 2008.
26. Nayak RC, Chang KH, Singh AK, Kotliar M, Desai M, Wellendorf AM, Wunderlich M, Bartram J, Mizukawa B, Cuadrado M, *et al*: Nuclear Vav3 is required for polycomb repression complex-1 activity in B-cell lymphoblastic leukemogenesis. Nat Commun 13: 3056, 2022.
27. Baxter PA, Lin Q, Mao H, Kogiso M, Zhao X, Liu Z, Huang Y, Voicu H, Gurusiddappa S, Su JM, *et al*: Silencing BMI1 eliminates tumor formation of pediatric glioma CD133+ cells not by affecting known targets but by down-regulating a novel set of core genes. Acta Neuropathol Commun 2: 160, 2014.
28. Liang J, Wang P, Xie S, Wang W, Zhou X, Hu J, Shi Q, Zhang X and Yu R: Bmi-1 regulates the migration and invasion of glioma cells through p16. Cell Biol Int 39: 283-290, 2015.
29. Vora P, Seyfrid M, Venugopal C, Qazi MA, Salim SA, Isserlin R, Subapanditha M, O'Farrell E, Mahendram S, Singh M, *et al*: Bmi1 regulates human glioblastoma stem cells through activation of differential gene networks in CD133+ brain tumor initiating cells. J Neurooncol 143: 417-428, 2019.
30. Freire-Beneitez V, Pomella N, Millner TO, Dumas AA, Niklison-Chirou MV, Maniati E, Wang J, Rajeeve V, Cutillas P and Marino S: Elucidation of the BMI1 interactome identifies novel regulatory roles in glioblastoma. NAR Cancer 3: zcab009, 2021.
31. Crosas-Molist E, Samain R, Kohlhammer L, Orgaz JL, George SL, Maiques O, Barcelo J and Sanz-Moreno V: Rho GTPase signaling in cancer progression and dissemination. Physiol Rev 102: 455-510, 2022.
32. Mosaddeghzadeh N and Ahmadian MR: The RHO family GTPases: Mechanisms of regulation and signaling. Cells 10: 1831, 2021.
33. Karlsson R, Pedersen ED, Wang Z and Brakebusch C: Rho GTPase function in tumorigenesis. Biochim Biophys Acta 1796: 91-98, 2009.
34. Kwiatkowska A, Didier S, Fortin S, Chuang Y, White T, Berens ME, Rushing E, Eschbacher J, Tran NL, Chan A and Symons M: The small GTPase RhoG mediates glioblastoma cell invasion. Mol Cancer 11: 65, 2012.
35. Si W, Shen J, Zheng H and Fan W: The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenetics 11: 25, 2019.
36. Li Y, Xu J, Chen H, Bai J, Li S, Zhao Z, Shao T, Jiang T, Ren H, Kang C and Li X: Comprehensive analysis of the functional microRNA-mRNA regulatory network identifies miRNA signatures associated with glioma malignant progression. Nucleic Acids Res 41: e203, 2013.
37. Lian S, Shi R, Bai T, Liu Y, Miao W, Wang H, Liu X and Fan Y: Anti-miRNA-23a oligonucleotide suppresses glioma cells growth by targeting apoptotic protease activating factor-1. Curr Pharm Des 19: 6382-6389, 2013.
38. Sun G, Cao Y, Shi L, Sun L, Wang Y, Chen C, Wan Z, Fu L and You Y: Overexpressed miRNA-137 inhibits human glioma cells growth by targeting Rac1. Cancer Biother Radiopharm 28: 327-334, 2013.
39. Que T, Song Y, Liu Z, Zheng S, Long H, Li Z, Liu Y, Wang G, Liu Y, Zhou J, *et al*: Decreased miRNA-637 is an unfavorable prognosis marker and promotes glioma cell growth, migration and invasion via direct targeting Akt1. Oncogene 34: 4952-4963, 2015.
40. Zhang JF, Zhang JS, Zhao ZH, Yang PB, Ji SF, Li N, Shi QD, Tan J, Xu X, Xu CB and Zhao LY: MicroRNA-770 affects proliferation and cell cycle transition by directly targeting CDK8 in glioma. Cancer Cell Int 18: 195, 2018.

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