Spalt-Like Transcription Factor 1 (SALL1) Gene Expression Inhibits Cell Proliferation and Cell Migration of Human Glioma Cells Through the Wnt/β-Catenin Signaling Pathway

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Background: The spalt-like transcription factor 1 (SALL1) gene is a member of the Krüppel-associated box-containing zinc finger proteins (KRAB-ZFPs) and has been shown to modulate the onset and progression of human tumors. This study aimed to investigate the regulatory effects and mechanisms of SALL1 gene expression in human glioblastoma and glioma cells and tissue samples from patients with cerebral glioma.

Material/Methods: The human glioblastoma cell lines, LN229, U87-MG, U-251, U343, and the Hs683 glioma cell line were studied. The cell counting kit-8 (CCK-8) assay, cell cycle assay, wound-healing assay, transwell assay, Western blot, and quantitative real-time polymerase chain reaction (qRT-PCR) were used to evaluate cell proliferation, cell migration, and the cell cycle and expression of SALL1. Expression of SALL1 mRNA in 120 samples of cerebral glioma and 20 samples of normal brain were studied. Overall survival data from patients with cerebral glioma were analyzed.

Results: SALL1 expression was down-regulated in human glioblastoma and glioma cells and in cerebral glioma tissues. Down-regulation of SALL1 expression was associated with reduced overall survival. Overexpression of SALL1 was associated with inhibition of cell proliferation associated with cell cycle arrest at the G0/G1 phase. SALL1 inhibited cell migration by preventing epithelial-mesenchymal transition (EMT) and down-regulating the expression of stem cell markers. Reduced levels of β-catenin and downregulation of c-Myc and cyclin D1 and up-regulation of p21and p27 expression were associated with SALL1 expression.

Conclusions: In human glioblastoma cells and cerebral glioma tissues, SALL1 acted as a tumor suppressor gene by inhibiting Wnt/β-catenin signaling.

MeSH Keywords: Genes, Tumor Suppressor • Glioma • Wnt Signaling Pathway

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Background

Cerebral glioma has a spectrum of malignancy from low-grade to high-grade. High-grade cerebral glioma is associated with high patient mortality rates [1]. Despite advances in treatment modalities, including surgery, chemotherapy, and radiation therapy, the median overall survival rate for patients with cerebral glioma has been reported to be 14.3 months from initial diagnosis [2]. Despite recent studies, the therapeutic options for patients with cerebral glioma are still limited, due to lack of knowledge regarding the pathogenesis [3–5]. Because the mechanisms underlying the progression of cerebral glioma remain unclear, further studies are needed to identify novel diagnostic and prognostic biomarkers.

Zinc finger proteins (ZFPs) are main transcriptional regulators in the human genome and are mainly located in the 19q13 region [6]. The spalt-like transcription factor 1 (SALL1) gene is a member of the SALL gene family (SALL1 to SALL4) that encode Krüppel-associated box-containing zinc finger proteins (KRAB-ZFPs) and has been shown to modulate the onset and progression of human tumors by regulating gene expression, histodifferentiation, apoptosis, autophagy and stemness [6,7]. Several ZFPs, including ZIC1, ZNF932, and ZNF545 have been shown to behave as tumor suppressors in the progression of human cancer [8–10]. Other genes, including ZNFEB and KLF5, have been confirmed to act as oncogenes in multiple cancers [11,12]. The promoter of SALL1 has been reported to be frequently methylated in breast cancer and early-stage head and neck cancer [13,14]. However, the effects and mechanisms of SALL1 gene expression in cerebral glioma remain unknown.

Therefore, this study aimed to investigate the regulatory effects and mechanisms of SALL1 gene expression in human glioma cells and tissue samples from patients with cerebral glioma.

Material Methods

Cell culture and reagents

The human glioblastoma cell lines, LN229, U87-MG, U-251, U343, and the glioma cell line Hs683 were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. Normal human astrocytes (NHAs) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermofisher Scientific, Waltham, MA, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Tissue samples

This study was approved by the local Ethics Committee and included tissue samples from adult patients only. Informed consent was signed by patients at the beginning of the study. There were 120 glioma tissue samples and the 20 normal brain samples that were collected between 2008 and 2010 in the Department of Neurosurgery, the Second Affiliated Hospital of Harbin Medical University. None of the patients in this study had received chemotherapy or radiotherapy before tumor resection. The tissue specimens were formalin-fixed and embedded in paraffin wax. The histological grading of cerebral glioma was performed, based on the WHO classification, as low-grade (WHO I and II) and high-grade (WHO III and IV) [15,16].

Overexpression of the spalt-like transcription factor 1 (SALL1) gene

The longest transcript of the human gene of SALL1 was cloned into pcDNA-3.1 plasmids and then sequenced for validation by GenePharma Co. (Shanghai, China) [17]. U87-MG and U251 cells were cultured in 6-well plates at a density of 5×10⁴ cells/well, 24 h prior to transfection. Plasmids were transfected into glioblastoma and glioma cell lines using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 5 μg according to the manufacturer’s instructions. After 6–8 h transfection, the cells were cultured with the 10% fetal bovine serum (FBS) containing medium and then transferred to selection medium containing 400 μg/ml G418 (gentamicin) (Invitrogen, Carlsbad, CA, USA) for two weeks. The stable-expressed cells were harvested for quantitative real-time polymerase chain reaction (qRT-PCR) to determine the transfection efficiency or complete functional assays.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR analysis was done to detect SALL1 mRNA expression. Firstly, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was applied to isolate total RNA from tissue specimens or cell lines following the manufacturer’s recommendations [16]. For the measurement of SALL1 expression, total RNA was converted to complementary DNA (cDNA) using a Taq Reverse Transcription Kit, and then the synthesized cDNA underwent quantitative PCR with a PCR Kit (Invitrogen, Carlsbad, CA, USA). Then qPCR was conducted using a PrimeScript RT Reagent kit (Takara Bio, Inc, Otsu, Japan). qRT-PCR was amplified under amplification reaction cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) with GAPDH as an internal control for 32 cycles. The SALL1 expression was normalized to GAPDH. Relative gene expression was quantified using the 2^(-ΔΔCq) method. The experiments were repeated three times and primer sequences are listed in Tables 1 and 2.
Semi-quantitative RT-PCR

SALL1 RNA and cDNA were reverse transcribed, according to the method described above, and RT-PCR was performed as previously described [18]. All samples were treated in a 10 μl reaction mixture containing 2 μl of cDNA, and β-actin was amplified as a control. The PCR conditions were: 95°C for 5 min; 95°C for 30 s; 55°C for 30 s; 72°C for 30 s; 25°C for 10 min, with 32 cycles for SALL1 and 23 cycles for β-actin using Go-Taq (Promega, Madison, WI, USA) and performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

Table 1. The forward (F) and reverse (R) polymerase chain reaction (PCR) primers.

| Primer | Sequence (5’-3’) | Product size (bp) | PCR cycles | Annealing temperature (°C) |
|--------|------------------|-------------------|------------|---------------------------|
| STAT3-F | CCAATGGAATCAGCTACAGC | 236               | 32         | 55                        |
| STAT3-R | GCTGATAGAAGACATTGAGTC |                   |            |                           |
| ALDH1-F | GAAATGTCACTCTTCCAGGCAC | 171               | 32         | 55                        |
| ALDH1-R | ATCTCTTCTTCTACCTGGC |                   |            |                           |
| MAD2-F | AAATCGTGGCCGAGTTCTTC | 244               | 32         | 55                        |
| MAD2-R | CTTTCAGGACCTCACCAC |                   |            |                           |
| SOX2-F | AGGAAAGGCACTACAGCA |                   |            |                           |
| SOX2-R | TGGGAGGAAAGGTAACCAACAG | 281               | 32         | 55                        |
| KLF4-F | TCACATCTTCTCCAGTC |                   |            |                           |
| KLF4-R | TCCAGGAGATCGTTGAACTC | 262               | 32         | 55                        |
| ABCG2-F | CAGTGTCACAAGGAAACACC | 194               | 32         | 55                        |
| ABCG2-R | GAGACCAGGTTTCATGATCC |                   |            |                           |
| CD133-F | CCACAGATGTCTCTAAGGC | 187               | 32         | 55                        |
| CD133-R | TGGGATTCTAATGCTCCTG |                   |            |                           |
| β-actin-F | TCCTGTGGCAGTCCAGGAAACT | 315               | 40         | 60                        |
| β-actin-R | GAAGCATTTGGCGGTGACGAT |                   |            |                           |

F – forward; R – reverse.

Table 2. The forward (F) and reverse (R) primers used in quantitative real-time polymerase chain reaction (qRT-PCR).

| Primer | Sequence (5’-3’) | Product size (bp) | PCR cycles | Annealing temperature (°C) |
|--------|------------------|-------------------|------------|---------------------------|
| SALL1-F | TGATGTAGCCAGGATGT | 125               | 40         | 60                        |
| SALL1-R | AAAGAATTCAGCGCAGC |                   |            |                           |
| E-Cad-F | TACACTGCCAGGAGGCAGA | 103               | 40         | 60                        |
| E-Cad-R | TGCCACAGGTCTCCGAGGTA |                   |            |                           |
| Vimentin-F | GACCGAGTAAACCAAGCAGA | 150               | 40         | 60                        |
| Vimentin-R | GTCAACATCTGCTGAAAAAGAT |                   |            |                           |
| N-Cad-F | CGAATGATGAAAGGACCCATCC | 174               | 40         | 60                        |
| N-Cad-R | GAGGCCAATGCCTCTCATAGTCAA |                   |            |                           |

F – forward; R – reverse.

Semi-quantitative RT-PCR
SALL1 mRNA expression data

SALL1 mRNA expression data were obtained from the Oncomine database (Compendia Bioscience, Ann Arbor, MI), (www.oncomine.org/).

Cell viability assay

Cell proliferation of transfected glioblastoma cell lines U87-MG and U251 were measured with a cell counting kit-8 (CCK-8) assay. Cells (3,000 cells/well) were plated into 96-well plates after transfection for 48 h. Then, 20 μl of CCK-8 reagent per well were added into 96-well plates and incubated for 2 h after 24 h, 48 h, and 72 h. The assay was performed in triplicate.

Cell cycle assay

Flow cytometry analysis of the cell cycle was performed, as previously described [19]. Glioblastoma and glioma cells (1×10⁶ cells/well) were seeded into 6-well plates, and after being transfected with pcDNA3.1-SALL1 or pcDNA3.1-vector for 48 h. Cells were collected and washed twice using cold PBS, then fixed into ice-cold 70% ethanol for at least 24 h and stored at 4°C overnight. The cell cycle was analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Wound healing assay

Cell migration was evaluated using a wound-healing assay in vitro. Cells with stable expression of SALL1 or vector-transfected U87-MG and U251 cells were cultured in 6-well plates and grown until its reached 90–95% confluence, and then a 200 μl plastic tip was used to create wounds across each well. Migration images were taken every 12 h to observe the wound healing process by phase contrast microscopy using a Leica DMi4000B phase contrast microscope (Leica, Milton Keynes, Bucks, UK).

Transwell assay

Transwell chambers were used to investigate cell migration using Matrigel invasion assays, according to the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ, USA). In total, 5×10⁴ transfected cells were plated into the upper compartments of transwell chambers that were precoated with Matrigel. The lower compartments were covered with 500 μl of DMEM.
containing 20% FBS. After 24 h of incubation, the non-migrating cells in the upper chamber were removed with 30% ethyl alcohol, and the migrated cells were fixed in 4% paraformaldehyde and then stained with 0.5% crystal violet. Invaded cells were counted in five random fields by light microscopy at ×400 magnification. The experiments were performed in triplicate.

Western blot

After washing twice in cold PBS, total protein was extracted from transfected cells using RIPA lysis buffer (Upstate Biotechnology Inc., Lake Placid, NY, USA), following which the concentration of total protein was detected using a BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China) as described in the manufacturer’s instruction. Equal proteins (50 μg/lane of total extract) were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, St. Louis, MO, USA) and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Membranes blots were then incubated in PBS blocking buffer, containing 5% dried skimmed milk powder and 0.2% Tween-20 at room temperature for 3 h. The membranes were incubated in primary antibodies to SALL1 (1: 1000) (ab31526; Abcam, Cambridge, MA, USA), β-catenin (1: 1000) (ab32572; Abcam, Cambridge, MA, USA), active-β-catenin (1: 500) (#19807; Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (1: 2000) (ab134175; Abcam, Cambridge, MA, USA), c-Myc (#19807, 1: 2000, CST), p21 (1: 1000) (#2947; Cell Signaling Technology, Danvers, MA, USA), p27 (1: 1000) (#3686; Cell Signaling Technology, Danvers, MA, USA), STAT3 (1: 1000) (#9139; Cell Signaling Technology, Danvers, MA, USA), SOX2 (1: 1000) (#3579; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. GAPDH (1: 1000) was used as a negative control. After washing with PBS for 45 mins, the blot membranes were incubated with the secondary antibodies, including anti-rabbit IgG (1: 2000) (ab6721; Abcam, Cambridge, MA, USA) and anti-mouse IgG (1: 2000) (ab6728; Abcam, Cambridge, MA, USA) at room temperature for 1 h. The membranes were quantified by using the enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL, USA). All assays were performed in triplicate.
Statistical analysis

All data were analyzed using SPSS version 17.0 software (IBM, Chicago, IL, USA) and statistical analysis was performed by Student’s t-test. Analysis of variance (ANOVA) and a post hoc test was performed for comparison between groups. Patient survival data underwent Kaplan-Meier analysis. P<0.05 was considered to be statistically significant.

Results

SALL1 expression was downregulated in cerebral glioma tissue

Spalt-like transcription factor 1 (SALL1) gene expression was downregulated in the cerebral glioma tissues. Analysis of the relative expression of SALL1 mRNA from the Oncomine database showed low expression of SALL1 in cerebral glioma tissues compared with normal brain tissues (p<0.001) (Figure 1A, 1B). Also, SALL1 mRNA was examined in 120 glioma specimens (60 low-grade glioma, and 60 high-grade glioma) and 20 normal brain tissues. SALL1 expression in normal brain tissues was significantly increased compared with cerebral glioma tissues, and the expression of SALL1 in low-grade glioma tissues (WHO I and WHO II) was significantly greater than in high-grade glioma tissues (WHO III and WHO IV) (p<0.001) (Figure 1C).

No significant differences were found between demographic characteristics, gender, age, tumor location, and route of biopsy. Kaplan-Meier analysis suggested that downregulation of SALL1 was significantly associated with reduced overall survival in 120 cases of cerebral glioma (p<0.05) (Figure 1D).

SALL1 expression inhibited the proliferation of glioma cells in vitro

The expression of SALL1 mRNA was significantly lower in the glioblastoma and glioma cell lines than in the normal human astrocytes (NHAs) (p<0.001) (Figure 2A). The results from quantitative real-time polymerase chain reaction (qRT-PCR) showed significant transfection efficiency for SALL1 expression in U87-MG and U251 cells (p<0.001) (Figure 2B). The results of the cell counting kit-8 (CCK-8) assay showed that cell proliferation in the SALL1-transfected cells was significantly reduced at 48 h and 72 h (p<0.001) (Figure 2C).

SALL1 expression induced cell cycle arrest of glioma cells in vitro

Stable transfection of U87-MG and U251 cells significantly increased the proportion of cells in the G0-G1 phase by between 15–25% (p<0.001) (Figure 3), indicating that reduced cell survival associated with SALL1 expression was due to cell cycle
These findings indicated that SALL1 inhibited cell growth in cerebral glioma cells 

**SALL1 expression suppressed cell migration of glioma cells in vitro**

The results of the wound-healing assay and transwell migration assay showed that U87-MG and U251 cells that overexpressed SALL1 migrated significantly more slowly than the control cells at 48 h (p<0.01) (Figure 4A). Cell migration was significantly decreased in cells that overexpressed SALL1 (p<0.01) (Figure 4B).

**SALL1 expression and expression of epithelial-mesenchymal transition (EMT) markers by glioma cells in vitro**

The results of Western blot showed that SALL1 expression was associated with increased expression of the epithelial marker, E-cadherin, and reduced expression of vimentin and N-cadherin in cerebral glioma cell lines in vitro (Figure 5A). Also, quantitative real-time polymerase chain reaction (qRT-PCR) showed that E-cadherin mRNA expression was increased with SALL1 overexpression, while vimentin and N-cadherin were down-regulated (p <0.01) (Figure 5B). These results indicated that SALL1 expression affected the expression of markers of EMT in glioma cells in vitro. EMT is a key element in the control of the progression of cancer stem cells [21–23]. Also increased expression of SALL1 reduced some stem cell markers involved in the regulation of cell transcription [24,25].

**SALL1 regulated the Wnt signaling pathway in cerebral glioma cells in vitro**

Increased expression of SALL1 significantly inhibited the expression of activated β-catenin and its downstream target genes, the c-Myc oncogene and CCND1 (Figure 6A), and significantly upregulated p21 and p27 (p<0.01) (Figure 6A, 6B). These in vitro findings indicated that SALL1 might influence the progression of glioma by activating the Wnt/β-catenin signaling pathway.

Figure 4. Expression of spalt-like transcription factor 1 (SALL1) gene suppressed cell migration in glioma cells in vitro.

(A) The migration of U87-MG and U251 glioma cells were decreased by overexpression of SALL1. *** p<0.01.

(B) The inhibition of cell migration of U87-MG and U251 cells by overexpression of SALL1. *** p<0.001.
Discussion

Previous studies have shown that the spalt-like transcription factor 1 (SALL1) gene is frequently silenced in multiple cancers by promoter methylation and that it acts as a functional tumor suppressor gene [14,15]. SALL1 expression is downregulated in breast cancer [14], and increased expression of SALL1 is associated with tumor proliferation and migration [26,27]. However, the expression and role of SALL1 in cerebral glioma was not previously investigated.

In the present study, interrogation of the Oncomine database supported the SALL1 gene was significantly downregulated in cerebral glioma tissue compared with normal brain tissue. The findings of this study showed that SALL1 expression was reduced in cerebral glioma, and showed the least expression in high-grade cerebral glioma (WHO III and WHO IV). Also, the findings showed that down-regulation of SALL1 was associated with reduced prognosis in patients with cerebral glioma, indicating that SALL1 might act as a tumor suppressor in cerebral glioma.

Zinc finger proteins (ZFPs) have previously been shown to be associated with biological functions associated with human cancer, including cell proliferation, apoptosis, and cell migration [11–13]. In this study, the effects of SALL1 gene expression were investigated in the human glioblastoma cell lines, U87-MG and U251, and reduced cell viability associated with
SALL1 expression was induced by G0/G1 cell cycle arrest. Also, SALL1 gene expression in U87-MG and U251 reduced the migratory capacity of glioma cells in vitro. These findings indicated that SALL1 expression was associated with aggressive behavior of cerebral glioma.

The process of epithelial-mesenchymal transition (EMT) confers stem cell properties that involve molecular changes, increased cell motility, and decreased cell-cell junctions and adhesion [28–30]. Cancer stem cells represent undifferentiated cancer cells, and EMT maintains stem cell features. Patients with cerebral glioma may have a poor prognosis, which has been shown to be associated with the presence of glioma stem cells [31,32]. Therefore, it is important to identify glioma stem cells and glioma stem cell-associated diagnostic targets to improve patient outcome in cerebral glioma. This study showed that increased expression of SALL1 upregulated epithelial markers, such as E-cadherin, and down-regulated mesenchymal markers, indicating that SALL1 gene expression negatively regulated EMT. Investigation of some downstream cancer stem cell markers showed that SALL1 down-regulated stem cell markers, such as STAT3. Therefore, the SALL1 gene might participate in the progression of cancer stem cells, which may be involved in glioma [24].

Previous studies have shown that ZFPs, including ZNF382, ZNF191, and ZIC2 play a vital role in cell metabolism, cell differentiation, and apoptosis through regulating the Wnt/β-catenin pathway [11–13]. ZNF382 undergoes frequent methylation and inhibits malignant behavior in esophageal squamous cell carcinoma by inhibiting the Wnt/β-catenin signaling pathway [33]. ZNF191 can directly bind to the beta-catenin gene (CTNNB1) promoter and activates the transcription expression of β-catenin and its downstream target genes, such as cyclin D1, in hepatoma cell lines [34]. In colorectal carcinoma, ZNF545 has been shown to be inactivated due to promoter methylation and regulated cell proliferation, apoptosis, and metastasis by its effects on the Wnt/β-catenin, PI3K/AKT, and MAPK/ERK signaling pathways [28]. The findings from a recent study showed that zinc finger protein S21 (Zfp521) regulated osteogenic differentiation of mesenchymal stem cells through the suppression of the Wnt/β-catenin signaling pathway [35]. Also, several previously published studies have shown that the Wnt signaling pathway is involved in the progression of glioma [36–40]. However, these previous studies did not elucidate the mechanisms of regulation of the Wnt/β-catenin pathways by ZFPs. Whether SALL1 functions by similar mechanisms that act on the Wnt/β-catenin pathway in cerebral glioma remains unknown.

Figure 6. Expression of spalt-like transcription factor 1 (SALL1) gene downregulated Wnt signaling in U87-MG and U251 glioma cells in vitro. (A) Western blot of β-catenin and its downstream targets in U87-MG and U251 glioma cells. (B) Relative protein levels of β-catenin, activated β-catenin, and its downstream targets. *** p<0.001.
The findings of this study showed that overexpression of SALL1 caused a significant decrease in inactivated β-catenin and its downstream target genes in glioblastoma and glioma cell lines in vitro. In the presence of Wnt signaling, cytoplasmic β-catenin protein is activated and accumulates in the cytoplasm and translocates to the nucleus where it activates the transcription of Wnt downstream target genes, including cyclin D1, c-Myc, and EMT markers [38–40]. The findings of the present study showed that SALL1 also suppressed the expression of c-Myc and cyclin D1, which are downstream targets of Wnts. This signaling pathway is likely to have contributed to the tumor suppressive effect of SALL1, as c-Myc is an important oncogenic transcription factor [41]. Cell cycle-associated markers, including p21 and p27, were upregulated in this study and expression of the SALL1 gene antagonized Wnt/β-catenin signaling in cerebral glioma cells.

Conclusions

The findings of this study showed that spalt-like transcription factor 1 (SALL1) gene might act as a functional tumor suppressor gene in cerebral glioma as the expression of SALL1 suppressed glioma cell proliferation and migration in vitro by inhibiting epithelial-mesenchymal transition (EMT) and antagonizing the Wnt/β-catenin signaling pathway.

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