STC1 regulates glioblastoma migration and invasion via the TGF-β/SMAD4 signaling pathway

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Abstract. Stanniocalcin-1 (STC1) is involved in cancer progression; however, the function of STC1 in glioblastoma remains unknown. In the present study, the expression levels of STC1 protein in glioblastoma were detected using immunohistochemistry. The expression levels of STC1, SMAD2/3 and SMAD4 proteins, following silencing of STC1, were assessed via western blotting. Edu and Transwell assays were performed to determine the proliferation and migration ability of the cells. The mRNA expression levels of STC1, SMAD4 and microRNA (miR)-34a were determined using quantitative PCR. The expression levels of STC1 were increased in glioblastoma tissues. STC1 revealed a significant association with poor outcome in patients with glioblastoma (P<0.05). The proliferation and invasion abilities were repressed in LN229 cells infected with LV3-shSTC1-1 and LV3-shSTC1-2 compared with LV3-NC. By contrast, the proliferation and invasion abilities were increased in T98G cells infected with LV5-STC1 compared with LV5-NC (P<0.05). The expression levels of STC1, SMAD2/3 and SMAD4 were decreased in LN229 cells infected with LV3-shSTC1-1 and LV3-shSTC1-2 compared with LV3-NC. However, the expression levels of STC1, SMAD2/3 and SMAD4 were elevated in T98G cells infected with LV5-STC1 compared with LV5-NC. The expression levels of miR-34a were decreased following silencing of STC1 (P<0.05). The expression levels of SMAD4 were decreased when transfected with miR-34a mimics (P<0.05). The luciferase activity of the wild-type 3’untranslated region of SMAD4 was decreased following transfection with miR-34a mimics (P<0.05). Silencing of STC1 inhibited the growth of LN229 in vivo. In conclusion, STC1 expression levels were increased in the present study, and it was revealed that STC1 regulated glioblastoma malignancy. This phenotype was observed in the SMAD2/3 and SMAD4 pathways.

Introduction

Glioblastoma is one of the most common types of cancer and accounts for 46.1% of all glioma cases in 2015, with an incidence of ~3.20 per 100,000 individuals every year (1-3). In recent years, surgery and adjuvant chemotherapy have made great progress alongside the development of medical technology; however, the 5-year survival rate of patients with glioblastoma remains poor. The main cause of this poor prognosis is tumor invasion and metastasis (4-6), though the underlying molecular mechanism remains unclear; therefore, elucidating this molecular mechanism has important clinical significance.

Stanniocalcin-1 (STC1) is one of the most secreted glycoprotein hormone (7). It maintains calcium homeostasis in teleost fish (8,9). Some studies have reported that STC1 is expressed in human tissues, including the ovary, kidney, brain, lung, heart, muscle and skeletal tissues (10-14). In addition, STC1 has been demonstrated to be associated with angiogenesis, apoptosis and inflammation (15,16). Accumulating evidence has demonstrated that STC1 plays a crucial role in numerous different types of cancer. Aberrant expression of STC1 has been observed in human carcinoma samples, including those from colorectal cancer, hepatocellular carcinoma, breast cancer, lung adenocarcinoma and thyroid carcinomas (13,17-19). In liver cancer, high expression levels of STC1 result in decreased energy metabolism and, in turn, stimulate the AMPK pathway and inhibit p70S6K/p-rpS6 signaling (20). In cervical cancer, STC1 regulates cell apoptosis via NF-κB phospho-P65 Ser536 (21). Another study demonstrated that the elevated expression levels of STC1 resulted in decreased energy metabolism and, in turn, stimulate the AMPK pathway and inhibit p70S6K/p-rpS6 signaling (20). In cervical cancer, STC1 regulates cell apoptosis via NF-κB phospho-P65 Ser536 (21). Another study demonstrated that the elevated expression levels of STC1 promoted the invasion of triple negative breast cancer cells through the activation of the c-Jun N-terminal kinase (JNK)/c-Jun signaling pathway (22). However, the expression and function of STC1 has not yet been elucidated in glioblastoma.

In the present study, the expression level of STC1 was examined in glioblastoma tissues and cell lines. Additionally, the function and the mechanisms underlying STC1 were investigated in this type of cancer.

Key words: SMAD2/3, stanniocalcin-1, SMAD4, microRNA-34a, glioblastoma
Materials and methods

Tissues. The patients were admitted to Chongqing Red Cross Hospital between January 2014 and January 2017 and diagnosed with glioblastoma by a pathologist. All patients underwent lesion resection and all patients with glioma received a postoperative pathological diagnosis. The present study was approved by the Ethics Committee of Chongqing Red Cross Hospital, and written informed consent was obtained from all patients. Samples from 80 cases of glioblastoma and adjacent non-cancerous tissue were obtained. The distance between the glioblastoma and normal tissue was 2 cm. The patients included 38 women and 42 men, with an age range of 16-62 years. Patients with glioblastoma had their diagnosis confirmed by pathological examination. Normal brain tissue was derived from surgical treatment of benign brain lesions, excluding patients with inflammation and malignant tumors.

Immunohistochemistry (IHC) staining. The expression of STC1 in glioblastoma was detected via IHC staining as previously described (23). Tissue were fixed with 10% formalin at room temperature for 48 h, paraffin-embedded (thickness, 4 µm) tissue sections were deparaffinized at room temperature for 1 h, washed with xylene at room temperature for 15 min and rehydrated in a descending alcohol series for 20 min. Following antigen retrieval and inactivation of endogenous peroxidase, the sections were blocked with 10% goat serum following antigen retrieval and inactivation of endogenous antibody anti-rabbit immunoglobulin G conjugated to horse (cat. no. ab229477; Abcam), followed by incubation with secondary antibody anti-STC1 primary antibody at 37˚C for 2 h (1:200; catalog no. sc-6101; Santa Cruz). After washing with PBS, the sections were incubated in horseradish enzyme-labeled chain avidin solution for 15 min. After washing with PBS, the sections were incubated in horseradish enzyme-labeled chain avidin solution for 15 min at 37°C and washed again. Subsequently, the proteins were visualized using 3,3'-diaminobenzidine (cat. no. ZLI-9017; OriGene Technologies, Inc.). Sections were observed with a light microscope (magnification, x20). The STC1-positive cells were scored as follows: 0 (negative intensity), 1 (weak intensity), 2 (moderate intensity) or 3 (strong intensity). The percentage of STC1-positive cells was scored as follows: 0, 1, 2, 3 and 4 from no cytoplasm expression, and 1-25, 26-50, 51-75, and 76-100% cytoplasm expression, respectively (23). ImagePro Plus 6.0 (Media Cybernetics, Inc.) was used to analyze the stained sections. The final STC1 staining score was obtained by multiplying the intensity and percentage score. The low expression was defined as staining score ≤6, while the high expression was defined as staining score >6.

Cell culture and transfection. The U251 cell line was purchased from the Chinese Academy of Sciences Cell Library (313IC000100700058). LN229 [American Type Culture Collection (ATCC® CRL-2611) and T98G (ATCC® CRL-1690™)] were purchased from the ATCC. The U87 cell line (Chinese Academy of Sciences Cell Library) represents glioblastoma of unknown origin. U251 cells were purchased from The Chinese Academy of Sciences Cell Library. The human astrocyte cell lines LN229 and T98G were purchased from ATCC. The four glioma cell lines were cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO2. Small interfering (si)RNAs, miRNA mimics and inhibitor were purchased from Shanghai GenePharma Co., Ltd. After 48 h, cells were collected for subsequent experimentation. The sequences of miR-34a-5p inhibitor, miR-34a-5p mimic and negative control were used as follows: miR-34a-5p inhibitor, 5'-ACAACCAGCUAAGCACUGCA-3'; miR-34a-5p mimics, 5'-UGGCGAGUCUUCAGCGUUGU-3'; negative control, 5'-UUCCUGCAACGUGUCAGTTT-3'. The concentration of miRNA transfected was 5 nM. siRNAs were obtained from Shanghai GenePharma Co., Ltd. The siRNA sequences were as follows: STC1-1, 5'-GCAGCGAGCAUCACGCAGCAACA-3'; STC1-2, 5'-GAUCACACUUCACUCAAGC-3'; negative control siRNA, 5'-UUCUCGAAGGUGUCAGGU-3'. Lentiviral vectors expressing shRNA-targeting STC1 (named LV3-shSTC1-1 and LV3-shSTC1-2) and STC1-lentiviral expression vector (named LV5-STC1) were obtained from Gene pharm, Inc. The sequences of the shRNA were the same as the siRNA sequences. In total, 10 nM siRNA was transfected into the cells. Cells were transfected using the siRNA-Mate transfection reagent (GenePharm, Inc.).

Luciferase reporter assay. Cells were transfected in six-well plates with 2 µg of a PReport plasmid (GenePharm, Inc.) containing the STC1 3'UTR including the binding site of miR-34a. Cells in each well were cotransfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) with 20 nM miR-34a mimics or negative control mimics. After 48 h, luciferase assays were performed using a dual-luciferase reporter assay system (Promega Corporation). Luciferase activity was normalized relative to Renilla luciferase activity.

Total RNA isolation and reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the cancer cells using RNA pure High-purity Total RNA Rapid Extraction kit (BioTeke Corporation; cat. no. RP1201) according to the manufacturer’s protocol. RT and qPCR kits (both from Bio-Rad Laboratories, Inc.) were used to determine the mRNA expression levels. The RT and qPCR reactions were performed as previously described (18). The RT reaction protocol was performed using three sequential incubations of 5 at 25°C, 30 min at 42°C and 5 min at 85°C. The thermocycling conditions were as follows: Denaturation at 95°C for 10 sec, 60°C for 20 sec, 72°C for 10 sec. qPCR kit (All-in-One qPCR Mix SYBR Green) and primers were purchased from GeneCopeia, Inc. The relative mRNA expression levels were calculated using the comparative quantification cycle 2^ΔΔCq method (18). GAPDH was used as an endogenous control to normalize the expression of the target genes. The primers for STC1 (cat. no. HQP017782) and GAPDH (cat. no. HQP064347) were purchased from GeneCopeia, Inc.

Western blot analysis. The expression of STC1, p-SMAD2/3, p-SMAD4 and GAPDH proteins was determined via western blotting as previously described (19-21), using STC1
(1:1,000; catalog no. ab229477), p-SMAD2/3 (1:1,000; catalog no. ab63399), SMAD4 (1:1,000; catalog no. ab191026), Phospho-SMAD4 (1:1,000; catalog no. PA5-64712) and GAPDH (1:1,000; catalog no. ab8245) antibodies (all from Abcam). The secondary antibody (1:5,000; anti-rabbit IgG, HRP-linked; cat. no. 7074) was purchased from Cell Signaling Technology, Inc. SuperBrite ECL western blot substrate and detection kit were purchased from AmyJet Scientific, Inc. The bands were analyzed using Image Lab (version 5.2; Bio-rad laboratories, Inc.).

EdU assay. The cellular proliferation ability was detected using an EdU assay, according to the manufacturer's protocol (24,25).

Cell invasion assays. Transwell invasion assays were performed as previously described (26,27). Then, the results were observed with a light microscope (magnification, x20).

Xenograft model. All procedures for the animal experiments were approved by The Committee on The Use and Care of Animals of The Chongqing Red Cross Hospital, and performed in accordance with the institutional guidelines. In total, 20 female, 6-week-old BALB/c nude mice (weight, 16 g) were purchased from the Laboratory Animal Center of Chongqing Red Cross Hospital. They were maintained in collective cages in an appropriate room with controlled temperature (22±2°C), humidity (60-70%) and a 12-h light/dark cycle, and were fed with standard mouse chow and water. The mice had free access to food and water. The cancer cells were infected with the indicated lentiviral vectors (MOI, 20) and injected (5x10^6 cells/mouse in 100 µl) subcutaneously into the left armpit. A total of 21 days later, the mice were sacrificed to confirm the presence of tumors and the established tumors were weighed. There was no sign of distress observed in the xenografted mice.

Bioinformatics analysis. Oncomine database (version 3.0; https://www.oncomine.org/) was used to analyze the glioblastoma-derived transcriptomes. The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) database was also investigated using the GEPIA (version 2) online tool (http://gepia.cancer-pku.cn/). StarBase software (version 2.0; http://starbase.sysu.edu.cn/starbase2/index.php) was used to investigate the interaction networks of different types of RNAs.

Statistical analysis. All statistical analyses were performed using SPSS software (version 19.0; SPSS, Inc.). Each experiment was performed in triplicate. The statistical analysis was performed using a Student's t-test or ANOVA followed by Scheffe's test. Data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant result.

Results

STC1 is associated with poor outcomes in patients with glioblastoma. In the present study, the expression patterns in glioblastoma were analyzed using the Oncomine database, and the expression levels of STC1 were increased in glioblastoma samples when compared with normal brain tissues in both Murat brain and Rickman brain datasets (28) (Fig. 1A and B). In addition, the expression patterns in glioblastoma were analyzed using TCGA database and GEPIA online tool.
expression levels of STC1 in glioblastoma were revealed to be higher than those in the normal brain tissues (Fig. 1C). The disease-free survival (DFS) and overall survival (OS) of STC1 genes was evaluated using the GEPIA online tool (http://gepia.cancer-pku.cn/). Low expression levels of STC1 mRNA were significantly associated with the optimal DFS and OS for all patients with glioblastoma (Fig. 1D and E).

**Increased expression of STC1 in glioblastoma tissues.** The expression patterns of STC1 in glioblastoma and normal brain tissue samples were investigated using IHC. STC1 was primarily localized in the cytoplasm (Fig. 2A-E). The staining intensity was significantly higher in the glioblastoma samples when compared with that in the normal brain samples (Fig. 2A-E and Table I). To analyze the association between STC1 expression and tumor grade, the cancer samples were divided into four groups. The expression of STC1 revealed a positive association with cancer grade (P<0.05; Table I), but not with the age of the patients (P>0.05; Table I). Furthermore, the STC1 expression levels in the U251, U87 and LN229 cell lines were higher than that in the T98G cell line (Fig. 2G).

**STC1 regulates proliferation and invasion in glioblastoma cells.** To investigate the role of STC1 in glioblastoma cells, STC1 expression was silenced in glioblastoma cells (Fig. 3A). Ectopic expression of STC1 led to successful overexpression (Fig. 3A). Furthermore, STC1 silencing inhibited the proliferation and invasion of LN229 cells (Fig. 3B-E), while the ectopic expression promoted the proliferation and invasion of T98G cells (Fig. 3B-E), thereby indicating that STC1 may promote glioblastoma malignancy.

**STC1 regulates transforming growth factor-β (TGF-β)/SMAD4 pathway.** To explore the potential mechanism of STC1 in glioblastoma, the association between STC1 and other genes was analyzed using the GEPIA online tool. Consequently, a positive correlation was established between STC1 and TGFBI, as determined by GEPIA online tool (Fig. 4A). In addition, STC1 was a potential competing endogenous (ce)RNA of SMAD4 as assessed by starBase software (Table S1). Thus, STC1 may be ascribed to the regulation of the TGF-β/SMAD4 pathway. Silencing STC1 inhibited the expression of p-SMAD2/3, SMAD4 and p-SMAD4 in LN229 cells (Fig. 4B), while the ectopic expression of STC1 promoted the expression of p-SMAD2/3, SMAD4 and p-SMAD4 in T98G cells (Fig. 4B).

**STC1 regulates miR-34a in a SMAD4-dependent manner.** The present study revealed that STC1 is a potential ceRNA
Table I. Association between STC1 expression and clinicopathological characteristics in 40 patients with glioblastoma tissues.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients, n (n=80)</th>
<th>STC-1 expression level</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Low, n (%)</td>
<td>High, n (%)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>16 (44.44)</td>
<td>20 (55.56)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>≥50</td>
<td>21 (47.73)</td>
<td>23 (52.27)</td>
<td></td>
</tr>
<tr>
<td>Tissue type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>30 (75.00)</td>
<td>10 (25.00)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cancerous</td>
<td>7 (17.50)</td>
<td>33 (82.50)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3 (37.50)</td>
<td>5 (62.50)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>2</td>
<td>2 (22.22)</td>
<td>7 (77.78)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 (8.33)</td>
<td>11 (91.67)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1 (9.09)</td>
<td>10 (90.91)</td>
<td></td>
</tr>
</tbody>
</table>

*Grades 3-4 vs. grades 1-2. STC1, stanniocalcin-1.

Figure 3. Cellular proliferation and invasion, determined via EdU and Transwell assays. (A) Knockdown of STC1 inhibited the expression level of STC1 in LN229 cells and overexpression of STC1 promoted the expression level of STC1 in T98G cells. (B) Knockdown of STC1 inhibited the proliferation in LN229 cells and overexpression of STC1 promoted proliferation in T98G cells. (C) Quantification of EdU-positive cells. (D) Knockdown of STC1 inhibited the migration of in LN229 cells and Overexpression of STC1 promoted invasion in T98G cells. (E) Quantification of migration assay. Error bars represent the standard error. *P<0.05. STC1, stanniocalcin-1.
of SMAD4 that competes for multiple miRNAs (including miR-130ac, miR-15abc and miR-34ac) (http://starbase.sysu.edu.cn/starbase2/index.php).

It was observed that the expression levels of miR-34a and miR-34c were significantly increased in LN229 cells infected with LV3-shSTC1-1 or LV3-shSTC1-2 when compared with LV3-NC (Fig. 5A). The expression levels of miR-34a and miR-34c were suppressed in T98G cells infected with LV5-STC1 compared with LV5-nc (Fig. 5B). The expression levels of miR-34a were significantly increased when transfected with miR-34a mimics. By contrast, the expression of miR-34a was significantly decreased when transfected with miR-34a inhibitors (Fig. 5C). The expression levels of STC1 and SMAD4 were decreased when transfected with miR-34a mimics in LN229 cells (Fig. 5D). Conversely, the expression of STC1 and SMAD4 was increased when transfected with miR-34a inhibitors (Fig. 5D). In addition, the silencing of STC1 inhibited the expression of SMAD4. However, this regulation was abrogated when the cells were transfected with miR-34a inhibitors (Fig. 5E). The dual-luciferase reporter gene assay confirmed that STC1 and SMAD4 were the direct targets of miR-34a (Fig. 5F). These data indicated that STC1 regulated miR-34a in a SMAD4-dependent manner.

**Discussion**

In the present study, increased STC1 expression levels were observed in glioblastoma tissues. In addition, the silencing of
STC1 inhibited glioblastoma cell malignancy. STC1 is known to regulate the TGF-β/SMad4 pathway. The low expression level of STC1 mRNA was significantly associated with optimal DFS and OS for all patients with glioblastoma. Therefore, STC1 is a potential therapeutic target for glioblastoma and an independent predictor of prognosis.

A number of studies have demonstrated that STC1 plays a major role in cellular proliferation and apoptosis. Another study found that STC1 inhibited cellular apoptosis in colorectal cancer, ovarian cancer, breast cancer, thyroid cancer and glioma tumor (12,29-31). STC1 regulated the proliferation and invasion of cervical cancer cells via the NF-κB pathway (21). Another study demonstrated that the elevated expression levels of STC-1 promoted the invasion of triple negative breast cancer cells through the activation of the JNK/c-Jun signaling pathway (22). In the present study, increased expression levels of STC1 were revealed in glioblastoma tissues. In addition, silencing of STC1 inhibited glioblastoma malignancy. The data revealed that increased expression levels of STC1 in glioblastoma may promote the progression of glioblastoma.

SMAD4 is a mediator of the TGF-β-signaling pathway. High expression levels of SMAD4 result in upregulated expression of matrix metalloproteinase 9 and promote the progression of...
In C6 glioma cells, miR-124 inhibits proliferation by directly targeting SMAD4 (33). In the present study, it was observed that STC1 regulated the expression of SMAD2/3 and SMAD4, indicating that it may regulate the TGF-β/SMAD4 pathway.

By acting as ceRNAs, miRNAs are able to regulate mRNAs, long non-coding (lnc)RNAs and circular RNAs (34). Starbase is a network database used to predict mRNA or lncRNA regulation via miRNA (35,36). In the present study, it was predicted that STC-1 acted as a ceRNA of SMAD4 via miR-34a. This was confirmed when investigations revealed that the mutual regulation between STC1 and SMAD4 was dependent on miR-34a.

miR-34a was associated with the proliferation and apoptosis of meningioma cells. In addition, SMAD4 was identified as a direct target for miR-34a (37). miR-34a represses the endometrial cancer cell invasion and sphere formation (38). miR-34a is also a multi-drug-resistant molecule and a prognostic indicator of breast cancer, which may regulate drug-resistant breast cancer (39). Thus, miR-34a may act as a tumor promoter. In the present study it was observed that STC1 and SMAD2 were direct targets of miR-34a. STC1 regulated SMAD2 through miR-34a. These data indicated that STC1 may promote glioblastoma malignancy. miR-34a was also involved in the TGF-β1 pathway. TGF-β1 was secreted by tumor-associated macrophages, and promoted colorectal cancer cell proliferation and invasion by regulating the miR-34a/vascular endothelial growth factor axis (40). The present study demonstrated that STC1 regulates the TGF-β/SMAD4 pathway. STC1 was a direct target of miR-34a. Thus, the miR-34a/STC1 axis promoted cancer progression via the TGF-β/SMAD4 pathway.

In conclusion, aberrant expression levels of STC1 were observed in glioblastoma tissues in the present study. In addition, the silencing of STC1 inhibited glioblastoma malignancy; these biological effects were associated with the TGF-β/SMAD4 pathway.

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Availability of data and materials

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

Authors’ contributions

YX designed and performed the experiments. QW analyzed the data and wrote the article.
Ethics approval and consent to participate
The present study was approved by the Ethics Committee of The Chongqing Red Cross Hospital, and written informed consent was obtained from all patients. All procedures for animal experiments were approved by the Committee on the Use and Care of Animals of The Chongqing Red Cross Hospital, and performed in accordance with the institution guidelines.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


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