Stanniocalcin-1 promotes cell proliferation, chemoresistance and metastasis in hypoxic gastric cancer cells via Bcl-2

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Abstract. Gastric cancer (GC) is one of the most lethal diseases worldwide, but the mechanism of GC development remains elusive. In the present study, the roles of stanniocalcin-1 (STC1) in GC were investigated. It was demonstrated that overexpression of STC1 mRNA and protein were associated with poor survival of patients with GC. The expression of STC1 was enhanced in hypoxic GC cells and overexpression of STC1 facilitated cell proliferation in hypoxia but not in normoxia. Furthermore, STC1 promoted chemoresistance, migration and invasion in hypoxia. Upregulating the expression of STC1 enhanced the expression of B cell lymphoma (Bcl)-2, neural-cadherin and matrix metalloproteinase-2, whereas it reduced the levels of cytochrome c, cleaved-caspase-9, cleaved-caspase-3 and epithelial-cadherin. However, downregulation of STC1 altered the expression of these proteins in the opposite direction. Furthermore, disturbing the expression of Bcl-2 partly reversed the changes to these proteins and also the pro-proliferation, anti-apoptosis and pro-invasion potential of STC1. In vivo experiments indicated that enhanced expression of STC1 promoted tumor growth and metastasis in mice. Collectively, the results indicated that STC1 may serve as an oncogenic role in hypoxic GC via dysregulating Bcl-2, indicating that STC1 may be a potential therapeutic target in the treatment of GC.

Introduction

Gastric cancer (GC) is one of the most lethal diseases in the world (1). Hypoxia is a general problem in solid tumors, including GC. Hypoxic cells undergo pro-survival alterations, including improved proliferation and invasion ability and enhanced resistance to chemotherapy (2,3). Therefore, actions to clarify the mechanisms and restrain the malignant behavior of hypoxic GC are not only vital but also urgent.

Stanniocalcin-1 (STC1), a glycoprotein involved in calcium/phosphate homeostasis, was demonstrated to be elevated in several types of carcinoma tissue compared with corresponding normal tissue (4-7). Notably, Fang et al (7) reported that overexpression of STC1 in tissue from GC was associated with a poor prognosis, but the mechanisms are unclear. Notably, the expression of STC1 could be induced by hypoxia (8,9). Therefore, for better control of GC, it is necessary to elucidate the roles of STC1 in hypoxic GC and identify proper therapeutic targets.

Materials and methods

Cell lines and cell culture. GC cell lines HGC27 (#TCHu22) and NCIN87 (#SCSP-534) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China); MKN28 (#3111C0001CCC000482) and MKN45 (#3111C0001CCC000229) cells were obtained from The National Infrastructure of Cell line Resource (Beijing, China); SNU601 cells were obtained from the Korean Cell Line Bank (Seoul, Korea); SNU216 cells were provided by the Medical College of Xiamen University (Xiamen, China).
Retroviral packaging cells (Phoenix amphotropic cells) were gifts from Professor Gong Yang at Fudan University Shanghai Cancer Center (Shanghai, China). Cells were cultured in Minimum Essential Medium (cat. no. 11095-080; HGC27) or RPMI1640 medium (cat. no. 12633-012) containing 10% fetal bovine serum (cat. no. 10099-141; all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 units/ml penicillin and 100 µg/ml streptomycin. To simulate hypoxic environment in vitro, cells were incubated with 100 µM cobalt chloride (CoCl2; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) for at least 48 h according to different procedures. All cells were cultured or treated in a humid atmosphere at 37°C with 5% CO2 and 95% air.

It has been reported that MKN28, a kind of gastric tubular adenocarcinoma, was contaminated with MKN74, another gastric tubular adenocarcinoma cell line (12). However, throughout the study MKN28 was used, and the results are repeatable.

**Cell transfection and viral infection.** The retroviral plasmids carrying STC1 cDNA and the empty vector (Vec), small hairpin RNA (shRNA) against the open reading frame of STC1 mRNA and the scrambled shRNA (Scr) were provided by Professor Gong Yang. The cells were infected as described previously (4). SNU601-STC1 cells were transfected with either 50 nM Bcl-2-targeting small interfering (si)RNA (Bcl2i; targeting sequence, CAGGACCTGCCGCTGCAGAC) (13) or scrambled negative controls (NCi; target sequence, AAT) or (Ig)G or goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (cat. nos. 7076 and 7074, respectively; Sigma-Aldrich; Merck KGaA), X-linked inhibitor of apoptosis protein (XIAP; cat. no. 20455; 1:1,000) and β-actin (cat. no. sc-4970; 1:1,000; both Cell Signaling Technology, Inc., Danvers, MA, USA). β-Actin was used as loading control. The secondary antibodies were horse anti-mouse immunoglobulin (Ig)G or goat anti-rabbit IgG conjugated to hors eradish peroxidase (HRP) (cat. nos. 7076 and 7074, respectively; 1:2,000; Cell Signaling Technology, Inc.). Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500) was purchased from Merck KGaA. The integrated density of each band was normalized to the density of corresponding β-actin.

**Growth curve assay.** Growth curves were measured by MTT assay as previously described (14). To compare cell proliferation ability under hypoxia or normoxia, cells were cultured with or without CoCl2. The medium were refreshed at the 4th day. Data were collected at 1, 3, 5, and 7 days by measuring the absorbance at 490 nm.

**Cisplatin treatment.** Cells were treated with cisplatin (Hansoh Pharma, Lianyungang, Jiangsu, China) and CoCl2 for 48 h at 37°C. The half-maximal inhibitory concentration (IC50) was measured by MTT assay according to procedures described previously (15). The concentration of cisplatin used included 1, 10, 100 and 1,000 µM for the primary screening for approximate ranges of IC50, and 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM for the final evaluation of IC50. For apoptosis analysis, cells were treated with cisplatin at the approximate concentration of IC50 (100 µM for SNU601 and 50 µM for MKN28) and CoCl2 for 48 h prior to flow cytometry. Apoptosis was assessed by Annexin V-PE-7AAD (cat. no. 559763; BD Pharmingen; BD Biosciences, San Jose, CA, USA) following the protocol described previously (15).

**Cell migration and invasion assay.** To evaluate cell migration, wound-healing assays were performed as previously described (16). The widths of the wounds were measured at 72 h following scratching. The 24-well Transwell chamber with 8-µm pore and Matrigel (Corning, Inc., Corning, NY, USA) for evaluating cell invasion as previously described (16). Cells were incubated with CoCl2 in the two assays.

**Xenograft tumors in nude mice.** A total of 20 mice (BALB/c athymic nude mice; age, 4-6 weeks; weight, 15-20 g; sex ratio, 1:1) were purchased from, and also treated and maintained in Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were maintained in the animal facility in individually ventilated cages in specific pathogen-free conditions in a temperature of 18-29°C and humidity of 40-70%, with a 12-h light/dark cycle, and received food and water ad libitum. The animal experiments were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Sciences and performed following institutional guidelines and protocols. To observe the effects of STC1 on tumor growth in vivo, SNU601-STC1 and SNU601-Vec cells were used to generate xenograft tumors on mice. A total of 8x106 cells were subcutaneously injected into each mouse (4 mice for each cell line). The longest diameter ‘a’ and the shortest diameter ‘b’ of tumors were measured and the tumor volume was calculated with the use of the following formula: Tumor volume (in mm3) = axb²x0.52 (14). To observe the effects of STC1 on peritoneal metastasis in vivo, 1x10⁷ cells were injected into the peritoneal cavity of every mouse (six mice for each cell line). When the volume of subcutaneous tumor reached 1,500 mm³, mice were sacrificed. All the subcutaneous tumor and peritoneal cancerous nodes were excised and weighed.

**Online data analysis.** Data from the large online public database Kaplan-Meier Plotter (n=876; http://kmplot.com/analysis/) was analyzed on the website with the three different probes (204595_s_at, 204596_s_at and 204597_x_at) using the auto select best cut-off (17).
Immunohistochemical (IHC) staining and analysis. IHC assay were performed on a tissue microarray containing samples from GC patients (HStm-Ade180Sur-06; Outdo Biotech Co., Ltd., Shanghai, China). All procedures were in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients received surgery between August 2008 and March 2009. Excluding patients older than 70 years, 66 patients (28-70 years old) with follow-up information remained in the cohort. The clinical characteristics of patients are presented in Table I. The pathological stage was evaluated according to 7th American Joint Committee on Cancer staging system (18).

STC1 protein expression was detected by IHC staining, and the adjacent section was stained with hematoxylin-eosin and evaluated by two pathologists. The staining and scoring system was performed as previously described (4). The primary antibody against STC1 (N15; sc-14346; Santa Cruz Biotechnology, Inc.) was applied at a dilution of 1:200 and incubated in a humid chamber at 4°C overnight. The staining intensity was evaluated as follows: Low, negative or ≤40% weak staining; High, >40% cells weak staining, or any cells with moderate or strong staining).

Statistical analysis. Data expressed as the mean ± standard deviation (SD) of three independently repeated experiments. Error bars in all graphs present the SD. Student’s t-test was used to evaluate the difference between every two different groups. A Pearson χ² test was used to evaluate the association between different clinicopathological characteristics of patients. A Kaplan-Meier log-rank test was used to analysis the survival of patients. P<0.05 was considered to indicate a statistically significant difference. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the analysis.

Results

Elevated STC1 is associated with poor survival in GC. To examine the potential role of STC1 in patients with GC, we first investigated the large online database Kaplan-Meier Plotter. It was demonstrated that with different probes, higher expression of STC1 mRNA was associated with worse overall survival (OS) and first progression (FP) in patients with GC (Fig. 1A-F).

Table I. Clinical characteristics of patients with different levels of stanniocalcin-1 protein in gastric cancer tissue.

<table>
<thead>
<tr>
<th>Features</th>
<th>Low Patients, n (%)</th>
<th>High Patients, n (%)</th>
<th>P-value</th>
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<tr>
<td>Total</td>
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<td>49</td>
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</tr>
<tr>
<td>Age, years</td>
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<td></td>
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<tr>
<td>&lt;60</td>
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<td>9</td>
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<td>Sex</td>
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<td>Female</td>
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<td>16</td>
<td></td>
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<tr>
<td>Histologic grade</td>
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<td></td>
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</tr>
<tr>
<td>&lt;III</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>≥III</td>
<td>8</td>
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<tr>
<td>Nerve/vascular invasion</td>
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<td></td>
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</tr>
<tr>
<td>Negative</td>
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<td>34</td>
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<td>Positive</td>
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<td>T stage</td>
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<tr>
<td>T1/2</td>
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<td>6</td>
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<tr>
<td>T3/4</td>
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<td>17</td>
<td>43</td>
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<tr>
<td>M1</td>
<td>0</td>
<td>6</td>
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<tr>
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<td>11</td>
<td>16</td>
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<tr>
<td>III/IV</td>
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T, tumor; N, node; M, metastasis; TNM, tumor-node-metastasis.
Figure 1. Overexpression of STC1 is associated with poor prognosis in patients with GC. (A-F) Kaplan-Meier analysis of the OS and FP of patients with GC according to STC1 mRNA level on http://kmplot.com/analysis/ using indicated probes (204596_s_at, 204595_s_at, 204597_s_at) with the auto select best cut-off. Black lines and red lines indicated low levels and high levels of STC1, respectively. (G) Kaplan-Meier analysis of the OS in patients with GC according to STC1 protein level. (H) Representative images of low (left) and high (right) levels of STC1 protein in GC tissues in immunohistochemistry analysis. OS, overall survival; GC, gastric cancer; FP, first progression; STC1, stanniocalcin-1; HR, hazard ratio.
To further validate the association between STC1 and prognosis, IHC staining was used to evaluate the expression of the STC1 protein in tissue samples from GC. The clinicopathological characteristics of patients are summarized in Table I. Higher expression of STC1 in cancer tissue was associated with worse OS (P=0.033; Fig. 1G and H). Additionally, correlation analysis indicated that higher levels of STC1 in cancer tissue was associated with poorer differentiation (P=0.030) and more advanced tumor node and metastasis stages (P=0.021; Table I).

Collectively, these results suggested that overexpression of STC1 was associated with poor prognosis in patients with GC.

Expression of STC1 is enhanced in hypoxic GC cells. To investigate the roles of STC1 in GC in vitro, the expression of STC1 in a set of GC cell lines was screened (Fig. 2A). In the cells which expressed the lowest level of STC1 (SNU601), STC1 cDNA and the vector control was overexpressed; while in the cells which expressed the highest level of STC1 (MKN28), the expression of STC1 was knocked down with shRNA (Fig. 2B).
CoCl₂ was used, which inhibits the activity of the key enzyme in the oxygen-sensing pathway (19), to stimulate hypoxia. Results from immunoblotting analysis demonstrated that the expression of HIF1-α, the well-established marker of hypoxia (20), increased in cells treated with CoCl₂, indicating that the hypoxic environment had been successfully mimicked in vitro (Fig. 2B). Furthermore, the levels of STC1 in hypoxic cells were always increased compared with the same cell lines in normoxic conditions (Fig. 2B), indicating that STC1 may serve certain roles in hypoxic cells.

**STC1 promotes cell proliferation and chemoresistance in hypoxia.** Previous studies demonstrated that STC1 promoted cell proliferation in a series of cancer cells (4,6,21). However, the effects of STC1 on cell growth in normoxic or hypoxic GC cells have not been reported, to the best of our knowledge. In the present study, in cells cultured with normal medium the growth curves of SNU601-STC1 cells and SNU601-Vec cells could not be distinguished clearly (Fig. 2C), as well as the curves of MKN28-STC1sh cells and MKN28-Scr cells (Fig. 2D). However, when cells were co-cultured with CoCl₂, SNU601-STC1 cells proliferated more compared with the SNU601-Vec cells (Fig. 2E), while MKN28-STC1sh cells grew more slowly than MKN28-Scr cells (Fig. 2F). The results indicated that overexpression of STC1 promoted cell proliferation in GC cells in hypoxia but not in normoxia.
The influence of STC1 on chemoresistance in GC was measured further and the influence of STC1 on the cell response to cisplatin in a hypoxic environment was focused on. Results from IC\textsubscript{50} (Fig. 2G) and flow cytometry (Fig. 2H-I) demonstrated that, in hypoxic SNU601-STC1 cells, the IC\textsubscript{50} of cisplatin was \ (~82 µM, and the proportions of late and total apoptotic cells were 16.9 and 25.0%, respectively. However, in hypoxic SNU601-Vec cells, the IC\textsubscript{50} of cisplatin was only 71 µM, and the ratio of cells in late and total apoptosis was 38.5 and 47.4%, respectively. Furthermore, compared with MKN28-Scr cells, the IC\textsubscript{50} of MKN28-STC1sh cells was decreased by 16%, and the percentage of late and total apoptotic cells was improved by 33 and 23%, respectively. Therefore, enhanced expression of STC1 facilitated the resistance to cisplatin treatment in hypoxic GC cells.

**STC1 accelerates hypoxia-induced cell migration and invasion.** To elucidate the roles of STC1 in cell migration in GC, wound-healing assays were performed in normoxia and hypoxia. At 72 h following scratching, in cells cultured without CoCl\textsubscript{2}, the widths of wounds in SNU601-STC1 cells were slightly narrower than in SNU601-Vec cells, and the widths in MKN28-STC1sh cells were wider compared with the MKN28-Scr cells but the change was not statistically significant (Fig. 3A and B). However, in cells treated with CoCl\textsubscript{2}, the wounds in SNU601-STC1 cells were almost healed, while those in SNU601-Vec cells remained large (Fig. 3C and D). Similarly, the gaps in hypoxic MKN28-STC1sh cells were obviously wider compared with the control cells (Fig. 3C and D). The results suggested that overexpression of STC1 promotes cell migration in hypoxia, but not in normoxia.

Therefore, the function of STC1 on cell invasion under hypoxic conditions was further investigated. At 72 h following seeding, there were 33% more cells invading through the gel in SNU601-STC1 cells compared within control cells, while there were 60% fewer cells moving into the bottom chamber in MKN28-STC1sh cells compared with in MKN28-Scr cells (Fig. 3E and F). Collectively, in hypoxic GC cells, higher levels of STC1 facilitated cell invasion.
**STC1 inhibits apoptosis and promotes invasion via Bcl-2.** To investigate the mechanisms of STC1-promoted malignant properties under hypoxic conditions, the expression of key proteins in cells treated with CoCl$_2$ was measured. As presented in Fig. 4A, compared with those in SNU601-Vec cells, the expression of Bcl-2 was increased, while the levels of cytochrome c, cleaved-caspase-9 and cleaved-caspase-3 were decreased in SNU601-STC1 cells. In contrast, compared with the MKN28-Scr cells, the level of Bcl-2 was decreased, but those of cytochrome c, cleaved-caspase-9 and cleaved-caspase-3 were increased in MKN28-STC1sh cells. On the other hand, compared with corresponding control cells, E-cadherin was downregulated, but N-cadherin and MMP2 were upregulated in SNU601-STC1 cells; whereas E-cadherin was upregulated, but N-cadherin and MMP2 were downregulated in MKN28-STC1sh cells. The expression of XIAP and MMP1 were not markedly changed according to the different expression of STC1 (data not shown). The results indicated that the expression of STC1 was positively associated with that of Bcl-2, N-cadherin and MMP2, but negatively associated with that of cytochrome c, cleaved-caspase-9, cleaved-caspase-3 and E-cadherin.

As Bcl-2 is a well-characterized and relatively upstream anti-apoptotic protein, the expression of Bcl-2 was artificially knocked-down with siRNA in SNU601-STC1 cells (Fig. 4A), and the cells' biological behavior was tested under hypoxic conditions. As presented in Fig. 4B-D, in SNU601-STC1-Bcl2i cells, cell proliferation was arrested, and the ratios of apoptotic cells treated with cisplatin were raised in SNU601-STC1-Bcl2i cells. Furthermore, under hypoxic conditions, the expression of cytochrome c, cleaved-caspase-9, and cleaved-caspase-3 were upregulated in SNU601-STC1-Bcl2i cells compared with those in control cells (Fig. 4A). As
Bcl-2 was also involved in the process of epithelial-mesen-
chymal transition (EMT) (13,22,23), the invasive ability of
SNU601-STC1-Bcl2i cells was also measured. The results
demonstrated that the numbers of cells invading through the
Transwell chamber were decreased, and the expression of
E-cadherin was increased, but those of N-cadherin and MMP2
were lower in hypoxic SNU601-STC1-Bcl2i cells compared
with in SNU601-STC1-NCi cells (Fig. 4C and E). Therefore,
downregulating the expression of Bcl-2 could significantly
reverse the aggressive properties induced by STC1.

These results suggest that, in hypoxic GC cells, STC1 may
facilitate cell proliferation, chemoresistance and metastasis
via Bcl-2.

**STC1 promotes tumor growth and metastasis in vivo.** To
further test the influence of STC1 in vivo, SNU601-STC1 cells
and SNU601-Vec cells were injected into nude mice.

As presented in Fig. 5A, the volumes of subcutaneous
tumors with SNU601-STC1 cells were increased compared
with SNU601-Vec cells, and the disparities increased with
time. Furthermore, at the endpoint of the experiments the
weight of SNU601-STC1 tumors was increased compared
with that of SNU601-Vec tumors (Fig. 5B). Therefore, the
results suggested that SNU601-STC1 tumors grow faster than
SNU601-Vec tumors.

Additionally the weight of peritoneal masses produced with
SNU601-STC1 cells was significantly heavier compared with
SNU601-Vec cells (Fig. 5C), indicating that SNU601-STC1
cells caused increased peritoneal metastasis compared with
SNU601-Vec cells. Furthermore, the malignant nodules in the
abdominal cavity generated with SNU601-STC1 cells were
greater in number and larger compared with SNU601-Vec
cells (Fig. 5D).

In short, the results implied that overexpression of STC1
promoted gastric tumor growth and metastasis in nude mice
in vivo.

**Discussion**

In the present study, the roles of STC1 in GC were illustrated.
It was demonstrated that the higher level of STC1 mRNA and
STC1 protein were associated with worse survival of patients
with GC. In vitro experiments identified that the expression of
STC1 is enhanced in hypoxic GC cells, and that overexpres-
sion of STC1 facilitated cell proliferation in hypoxia but not
in normoxia. Furthermore, STC1 promoted chemoresistance, migration and invasion in hypoxia. Additionally, under hypoxic conditions, the elevated expression of STC1 was associated with an enhanced expression of Bcl-2, N-cadherin and MMP2, and downregulated levels of cytochrome c, cleaved-caspase-9, cleaved-caspase-3, and E-cadherin. However, interfering with the expression of Bcl-2 increased the levels of cytochrome c, cleaved-caspase-9, cleaved-caspase-3 and E-cadherin, decreased the levels of N-cadherin and MMP2, and further restrained cell proliferation, chemoresistance and cell invasion in hypoxia. In vivo experiments also indicated that STC1 promotes gastric tumor growth and metastasis. Collectively, STC1 may promote GC development, chemoresistance and metastasis via Bcl-2 under hypoxic conditions (Fig. 6).

In patients with GC, a previous study reported that the high/moderate level of STC1 protein was associated with poor progression-free survival (7), but the effects of STC1 on OS has not been illustrated. In the present study, according to data in the Kaplan-Meier Plotter database, a higher level of STC1 mRNA was associated with shorter OS and FP. In another cohort or GC patients, it was demonstrated that a higher level of STC1 protein in cancer tissues was associated with poorer outcomes (9,26-29). In another study, higher STC1 mRNA and protein levels were associated with shorter OS and FP (24,25). In the present study, it was demonstrated that higher level of STC1 protein in cancer tissues was associated with poorer outcomes. In conclusion, the results suggested that under hypoxic conditions STC1 may promote metastasis via restraining the Bcl-2-adjusted EMT process in GC.

By reducing the expression of Bcl-2, STC1 may promote GC development, chemoresistance and metastasis under hypoxia, and STC1 and Bcl-2 may be considered as potential therapeutic targets in GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW, ZQ, MZ, WY, RH and GL performed the experiments in this study on cells and mice, the database analysis and IHC analysis. YW, XM and ZZ designed the experiments. YW drafted the manuscript. XM and ZZ edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Sciences (Beijing, China). The use of human tissues was approved by the Ethics Committee of Outdo Biotech Co., and the cooperating hospitals and written informed consent was obtained from all patients.

Patient consent for publication

Consent for the publication of the clinical and pathological data was obtained from all patients who were involved in the present study.
Competing interests

The authors declare that they have no competing interests.

References


