SHCBP1 Promotes the Progression of Esophageal Squamous Cell Carcinoma Via the TGFβ Pathway

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Abstract: Esophageal cancer (EC) is known as a type of common malignant tumor, with the incidence ranking eighth worldwide. Because of the high metastasis of advanced EC, the total survival rate has been quite low. Esophageal squamous cell carcinoma (ESCC) is a main type of EC. Targeted therapy for ESCC has become a new direction; however, newly therapeutic targets are also badly needed. Shc SH2 domain-binding protein (SHCBP1) is located on 16q11.2, which is a downstream protein of the Shc adaptor. SHCBP1 participates in the regulation of several physiological and pathologic processes, such as cytokinesis. Recent studies have found that SHCBP1 was abnormally upregulated in multiple types of tumors, such as breast cancer and liver cancer, and that it affects the proliferation and motility of cancer cells in vitro. However, it remains unclear whether SHCBP1 is related to the progression of EC. Herein, we found the upregulation of SHCBP1 in human EC tissues. Our findings further demonstrated that SHCBP1 expression was related to the clinical features of ESCC patients. We found that SHCBP1 depletion inhibited the proliferation and motility of ESCC cells via the transforming growth factor β pathway and that it suppressed the growth of tumors in mice. We, therefore, concluded that SHCBP1 could serve as a promising EC molecular target.

Key Words: esophageal squamous cell carcinoma (ESCC), Shc SH2 domain-binding protein (SHCBP1), proliferation, migration, TGFβ pathway

Esophageal cancer (EC) was known as a common malignant tumor, with its incidence ranking eighth worldwide.1 Esophageal cancer (EC) was known as a common malignant tumor, with its incidence ranking eighth worldwide.1,2 Most patients have developed to local advanced stage or have had distant metastasis when diagnosed; hence, the total survival rate of EC patients is quite low.3,4 EC pathologic type includes esophageal squamous cell carcinomas (ESCC), which is common in Asia, and esophageal adenocarcinoma, which is common in European and American countries.5 In decades, the recurrence rate of early post-operative ESCC patients still cannot be effectively controlled, and the survival period of middle and advanced patients after chemoradiotherapy has not been significantly improved, resulting in poor therapeutic effect.6,7 With the development of molecular biology in recent years, the study of ESCC-related genes is getting more and more in-depth.8,9 Targeted therapy for ESCC has become a new direction; however, new therapeutic targets are also badly needed.9,10

Shc SH2 domain-binding protein (SHCBP1), which is located on 16q11.2, is a member of the Shc adaptor downstream proteins.11 SHCBP1 participates in a variety of physiological and pathologic processes, such as cytokinesis.12 For example, SHCBP1 phosphorylation was a requirement for cleavage furrow separation.13 In addition, through proteome analysis, SHCBP1 was also thought as a central spindle binding partner.13 SHCBP1 also affects the development of T cells and mediates multiple signaling pathways, such as PI3K/AKT and TGF-beta 1/Smad pathways.14,15

Recent studies have found that SHCBP1 is upregulated in a variety of tumors, such as breast cancer and liver cancer, and affects the growth and migration of cancer cells in vitro.16,17 SHCBP1 could activate the MEK/ERK pathway and therefore promote the growth of hepatocellular carcinoma cells.17 In addition, SHCBP1 could promote the migration of synovial sarcoma cells through the TGF-beta 1/Smad signaling pathway.18 In lung cancer, the nuclear
localization of SHCBP1 phosphorylates and activates β-catenin, promoting tumor development. In addition, SHCBP1 expression could also activate the NF-κB pathway and promote the migration and invasion of glioma cells. Collectively, these results reveal that SHCBP1 is a potential tumor target gene. However, it remains unknown whether SHCBP1 affects the progression of EC.

Herein, we reported the abnormal upregulation of SHCBP1 in human ESCC tissues and cell lines. Through clinical feature comparison, we revealed SHCBP1 expression was correlated with metastasis and maximum diameter. We further found that SHCBP1 promotes the proliferation and motility of ESCC cells in vitro and that it induces tumor growth of ESCC cells in mice. Our findings further confirm that SHCBP1 promotes of Smad2/3 and ERK1/2 phosphorylation, which therefore contributes to ESCC progression. We, therefore, present SHCBP1 as a novel and promising ESCC molecular target.

MATERIALS AND METHODS

Antibodies, Plasmids, and Primers
All the antibodies used in this study are shown as follows:

| Antibody | Information |
|----------|-------------|
| SHCBP1   | 1:200 for IHC; 1:2000 for Immunoblot, SAB1307183, Sigma-Aldrich, MO |
| β-actin  | 1:2500, ab8227; Abcam, UK |
| p-Smad2  | 1:2500, ab53100; Abcam, UK |
| Smad2    | 1:2000, ab33875; Abcam, UK |
| p-Smad3  | 1:2500, ab52903; Abcam, UK |
| Smad3    | 1:2500, ab40854; Abcam, UK |
| p-ERK1/2 | 1:2500, ab214362; Abcam, UK |
| ERK1/2   | 1:1500, ab17942, Abcam, UK |
| GAPDH    | 1:20000; Abcam, UK |
| p-GAPDH  | 1:2500, ab13359; Abcam, UK |
| β-actin  | 1:2500, ab8227; Abcam, UK |
| p-ERK1/2 | 1:2500, ab214362; Abcam, UK |
| ERK1/2   | 1:1500, ab17942, Abcam, UK |

The quantitative real-time polymerase chain reaction (PCR) primer sequences of SHCBP1 are shown as follows:

| Primers | Sequences |
|---------|-----------|
| SHCBP1-FP | 5'-CCCTTCTGAGCAAGTCGAGG-3' |
| SHCBP1-RP | 5'-AACTGTTTCCCCACAAATCC-3' |
| GAPDH-FP | 5'-CGACCCACTTGTCAGCTCTA-3' |
| GAPDH-RP | 5'-GGTGGAGCACAGGGTGACCT-3' |

The shRNA plasmids (Ready-to-package AAV) of SHCBP1 were bought from Addgene.

Immunohistochemistry (IHC)
IHC assays were performed to detect the expression of SHCBP1 in human EC tissues. Tumor tissues were obtained from the First Affiliated Hospital of Shantou University Medical College. To detect the expression of SHCBP1 in human ESCC tissues, we conducted IHC assays. First, fresh tissues were fixed with 4% paraformaldehyde (PFA) overnight at room temperature. Thereafter, we dehydrated the tissues in ethanol, cleared them in xylene, and embedded them in paraffin blocks. Sections of 5 mm were cut. Next, we placed the slides in preheated solutions for 5 to 10 minutes and then treated them as previously described for antigen retrieval using a microwave, and this was then followed by 60-minute blocking with 2% bovine serum albumin. The slides were subsequently incubated with SHCBP1 antibodies for ~2 hours. Thereafter, we incubated the sections with horseradish peroxidase–targeted antibody for 40 minutes and used diaminobenzidine for 5-minute incubation.

The proportion of positive-staining cells was scored as follows: 0 for negative-staining cells; 1 meant 10% to 50% positive-staining cells, and 2 represented >50% positive-staining cells. The staining intensity was evaluated on a score of 0 (negative-level staining), 1 (low-level staining), and 2 (high-level staining). The expression levels of SHCBP1 were further examined on the basis of this staining index: staining intensity score plus positive tumor cell staining percentage score. Staining index 0 to 2 was considered low expression, while staining index 3 to 4 was considered high expression.

Cell Culture and Transfection
The 4 types of human ESCC cell lines, KYSE150, KYSE30, KYSE180, and KYSE450, were all bought from ATCC, and maintained in Dulbecco Modified Eagle Medium, supplemented with 10% of fetal bovine serum (FBS) in a 5% CO2 incubator at 37°C. The shRNAs were transduced into ESCC cells by lipofectamine 2000 (11668019; Invitrogen). Stable depleted ESCC cells were screened by lentivirus infection and used in animal assays.

Quantitative PCR Assay
Quantitative PCR assays were conducted to detect the mRNA levels of SHCBP1 in EC tumors and cell lines. Trizol (15596026; Invitrogen) was used to isolate mRNA from human ESCC cells. Subsequently, reverse-transcription was performed using the reverse-transcribed RNA by using reverse transcriptase kit (M1701; Promega). In addition, total mRNA was then reverse transcribed using a synthesis system. Quantitative PCR was conducted by the use of SYBR premix Ex Taq II kit (RR420A; Takara, Japan), and the mRNA expression of SHCBP1 was normalized to the mRNA of glyceraldehyde 3-phosphate dehydrogenase.

Immunoblot Assays
Immunoblot assays were conducted to detect the protein levels of SHCBP1 in EC tumors and cell lines. Proteins were lysed by RIPA buffer. Thereafter, proteins were isolated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) methods and were transferred onto the nitrocellulose (NC) membranes; the membranes were blocked by the use of 5% fat-free milk in TBST and incubated with antibodies to detect SHCBP1 etc., at room temperature for 2 hours. Afterward, NC membranes were washed using TBST buffer for 4 times and then the NC membranes were incubated with horseradish peroxidase–targeted antibodies for about 1 hour. After washing, signals were detected through enhanced chemiluminescence agent.
3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) Assays

MTT assays were conducted to detect the proliferation ability of ESCC cells. ESCC cells were first added into 96-well plates, transfected with control or SHCBP1 shRNA plasmids, and cultured for 48 hours. ESCC cells were incubated using MTT for 4 hours and washed with phosphate-buffered saline (PBS) twice. Thereafter, the OD562 value was detected using a microplate reader.

Bromodeoxyuridine (BrdU) Proliferation Assays

BrdU assays were conducted to detect the proliferation ability of ESCC cells. ESCC cells were incubated with 10 mmol/L BrdU (Sigma). After 1 hour, cells were fixed and analyzed by BrdU Cell Proliferation Assay Kit (K306-200; Biovision). The proportion of BrdU-positive nuclei in 8 random fields (500 total nuclei) was analyzed and determined for each group.

Colony-formation Assay

Colony-formation assays were conducted to detect the proliferation ability of ESCC cells. A total amount of 1000 cells was added into a 6-well plate, and they were transfected with shRNA plasmid and cultured in a 37°C, 5% CO2 incubator for 24 hours. After 2 weeks, ESCC cells were fixed with PFA for 30 minutes, treated with 0.2% crystal violet buffer at room temperature for 30 minutes, and washed out for 3 times. Thereafter, the colony numbers were manually counted.

Wound-healing Assays

Wound-healing assays were conducted to detect the migration ability of ESCC cells. KYSE180 cells were transfected with the shRNAs, cultured for 48 hours, and the wound was created with a 20 μL tip. Thereafter, the cells were washed, and the culture medium containing 10% FBS was used to stimulate healing. Photographs were taken at 0- and 24-hour timepoints, and the relative degree of wound closure was compared.

Transwell Assays

Transwell assays were conducted to detect the invasion ability of ESCC cells. ESCC cells were transfected with the indicated plasmids for 48 hours and then resuspended in medium without FBS. The upper chamber in filters (8.0 μm membrane) was coated with 20% matrigel (in RPMI-1640) and incubated for 30 minutes. About 10^5 cells in the medium were seeded into the upper chamber and stimulated to move toward the bottom chambers with 10% FBS. After 1 day, cells at the top were removed with cotton swabs, and the remaining cells were fixed using 4% PFA for 20 minutes, and they were stained with 0.2% crystal violet buffer for 30 minutes. Thereafter, the relative cell number was compared.

Tumor Growth Assays

Tumor growth assays were used to assess the tumor growth capacity in mice. All animal assays were approved by Medical Animal Care & Welfare Committee of Shantou University Medical College (Approval no. SUMC2017-170). For tumor growth assay, KYSE180 cells were stably infected with control or SHCBP1 shRNA lentivirus. Control or depletion cells were washed and resuspended in PBS buffer (2.0×10^7 cells/mL). Around 2.0×10^6 cells in 100 μL PBS were subcutaneously injected into athymic BALB/c nu/nu mice. After 2 weeks, tumor volume was measured every 3 days.

Statistics

GraphPad 6.0 was used for statistical analysis. All data were represented as mean ± SEM. In addition, the correlation analysis between clinical pathologic features and SHCBP1 expression was performed through χ2 analysis. Kaplan-Meier survival analysis were performed to evaluate the prognosis. The Student t test was used for statistical comparisons. ** indicates P-value < 0.01.

RESULTS

SHCBP1 is Abnormally Upregulated in Human ESCC Tissues

To evaluate the possible role of SHCBP1 in the progression of ESCC, we assessed its mRNA and protein levels in tumor tissues and normal tissues. A total of 40 ESCC patients were recruited in this study. Quantitative real-time PCR assays showed that the mRNA level of SHCBP1 was upregulated in tumor tissues, compared with the corresponding normal tissues (Fig. 1A). We then performed IHC assays to further confirm the expression difference in tumor and normal tissues. As we expected, we noticed the obvious upregulation of SHCBP1 in human ESCC tissues, whereas the normal tissues had lower SHCBP1 expression level (Fig. 1B).

Next, we detected the mRNA levels of SHCBP1 in human normal esophageal squamous epithelial cell (Het-1A) and ESCC cell lines, including KYSE150, KYSE30, KYSE180, and KYSE450. In line with the results above, we found SHCBP1 was significantly upregulated in ESCC cells compared with that observed in normal esophageal squamous epithelial cells (Fig. 1C). All together, these results cleared the abnormal high expression level of SHCBP1 in human ESCC cells and tissues.

SHCBP1 Expression is Associated With the Clinicopathologic Characteristics and the Prognosis of Patients With ESCC

According to the levels of SHCBP1 mRNA in tumor tissues of 40 patients with ESCC, the patients were divided into SHCBP1-low-expression or high-expression groups. Interestingly, 15 of the patients (50%) exhibited SHCBP1 high expression, and the remaining showed low expression (Table 1). We then performed clinical pathologic characteristics’ analysis to explore the role of SHCBP1 in cancer progression.

In brief, patient sex, age, differentiation, metastasis, and tumor size were assessed, respectively. We found there were no significance between SHCBP1 expression and clinical parameters such as patient sex (P = 0.749), age (P = 0.525), and differentiation (P = 0.490, Table 1). By comparison, we noticed SHCBP1 expression was obviously
correlated with clinicopathologic features including metastasis staging ($P=0.004^*$) and tumor size ($P=0.011^*$, Table 1). We, therefore, demonstrated that SHCBP1 expression levels were correlated with clinicopathologic characteristics of patients who underwent ESCC.

**Knockdown of SHCBP1 Blocked Proliferation of ESCC Cells**

It is well known that tumors are induced by excessive cell proliferation. We asked whether the effect of SHCBP1 on ESCC was due to the stimulation of cell proliferation.
First, we transfected the shRNA plasmid-targeted SHCBP1 into human ESCC cell line KYSE180 (the highest SHCBP1 expression level), to suppress the expression level of SHCBP1. The results of quantitative PCR assays showed that the transfection of SHCBP1 shRNA effectively decreased its expression level in KYSE180 cell (Fig. 2A). Consistent with quantitative PCR results, the immunoblot assays further proved the obvious decrease of SHCBP1 expression level in KYSE180 cell transfected with SHCBP1 shRNA plasmid (Fig. 2B).

Thereafter, we detected the effect of SHCBP1 on cell proliferation of ESCC by Brdu assays and colony formation. Brdu assays showed that SHCBP1 depletion resulted in a significant decreased proliferation capacity of KYSE180 cells (Fig. 2C). In addition, results indicated that the colony-formation ability was dramatically decreased by SHCBP1, confirmed by the decreased cell numbers (Fig. 2D). In conclusion, these results suggested that SHCBP1 promotes ESCC cell proliferation in vitro.

**Ablation of SHCBP1 Blocks Motility of ESCC Cells In Vitro**

Having come to know that SHCBP1 could promote ESCC cell proliferation, next, we conducted wound closure and transwell assays to assess the possible effects of SHCBP1 on the motility of ESCC cells. As was expected, our results revealed that SHCBP1 depletion remarkably inhibited the wound healing in KYSE180 cell (Fig. 3A). In addition, SHCBP1 depletion markedly blocked the invasion of KYSE180 cells, with an obviously dropped cell number (Fig. 3B), confirmed by transwell assays. Altogether, our results indicated that SHCBP1 stimulates cell motility of ESCC in vitro.

**Table 1. Relationship Between SHCBP1 and Clinicopathologic Parameters**

| Parameters              | No. Patients | Low (< Median) | High (≥ Median) | P   |
|-------------------------|--------------|----------------|-----------------|-----|
| N                       | 40           | 20             | 20              |     |
| Sex                     |              |                |                 |     |
| Male                    | 23           | 12             | 11              | 0.749|
| Female                  | 17           | 8              | 9               |     |
| Age (mean) (y)          |              |                |                 |     |
| >60                     | 18           | 10             | 8               | 0.525|
| <60                     | 22           | 10             | 12              |     |
| Differentiation         |              |                |                 |     |
| Well                    | 12           | 5              | 7               | 0.490|
| Moderate/poor           | 28           | 15             | 13              |     |
| Metastasis              |              |                |                 |     |
| Negative                | 21           | 15             | 6               | 0.004*|
| Positive                | 19           | 5              | 14              |     |
| Maximum diameter (cm)   |              |                |                 |     |
| ≤4.0                    | 22           | 15             | 7               | 0.011*|
| >4.0                    | 18           | 5              | 13              |     |

*P < 0.05.

SHCBP1 indicates SH2 domain-binding protein.

**DISCUSSION**

In the past few decades, more and more attention has been paid to targeted therapy for advanced EC. For example, targeted therapies for EGFR (ERBB1), ERBB2, and MET are under development. However, because advanced EC is highly invasive and metastatic, and the existing targeted therapy drugs still have little effect on improving the prognosis of patients, there is still an urgent need to develop new therapeutic targets. In this study, we collected 40 esophageal tumor tissues and...
the corresponding normal tissues and performed IHC assays to assess the expression level of SHCBP1. Interestingly, we found the obvious high expression of SHCBP1 in human ESCC tissues. Our data further confirmed that SHCBP1 expression was correlated with clinical pathologic features including metastasis and maximum diameter. We, therefore, thought SHCBP1 could act as a promising molecular target for ESCC treatment.

Performing BrdU proliferation assays and colony formation assays, we noticed that SHCBP1 ablation stimulated the inhibition of ESCC cells in vitro. Meanwhile, knockdown of SHCBP1 suppressed ESCC cell growth.
migration and invasion, which was confirmed by wound closure and transwell assays. Notably, we previously found the correlation between SHCBP1 expression and clinical characteristics including metastasis and maximum diameter, suggesting the potential effects of SHCBP1 on cancer cell proliferation and motility. Our data further revealed that SHCBP1 contributed to tumor growth of ESCC cells in vivo. Similarly, multiple studies indicated the involvement of SHCBP1 in cancer progression and development. SHCBP1 could contribute to cisplatin-induced apoptosis resistance and motility of lung cancer cells. Another study declared that SHCBP1 was upregulated in gastric cancer tissues and affected the proliferation of gastric cancer cells. In addition, SHCBP1 contributed to cancer metastasis. These studies, together with our findings, confirmed SHCBP1 was a key regulator in cancer progression.

Here, we also found that SHCBP1 affected the proliferation and motility of ESCC cells via the phosphorylation of Smad3 and Erk1/2. Our data further proved the key regulatory role of SHCBP1 via the TGFβ pathway during EC progression. SHCBP1, as was known, could mediate multiple signaling pathways, such as WNT, PI3K/AKT, TGF-β/Smad, and β-catenin pathways, which were all involved in the regulation of cancer progression. SHCBP1 contributed to cisplatin-induced apoptosis resistance via the WNT pathway. In addition, SHCBP1 could activate β-catenin signaling and promote the progression of non-small cell lung cancer. SHCBP1 also promotes migration and invasion in gliomas via the NF-κB signaling pathway, and in synovial sarcoma via TGFβ pathway. We, here, found that SHCBP1 contributed to the growth of ESCC via the effects on TGFβ pathway. Therefore, SHCBP1 has a variety of effects on tumors, and an in-depth understanding of the molecular mechanisms that affect tumors can be more effective in targeted therapy.

Notably, previous studies also confirmed that SHCBP1 could mediate midbody organization and cytokinesis completion. Meanwhile, the Aurora-B-mediated phosphorylation of SHCBP1 also affected cytokinetic furrow ingression. These findings suggest that SHCBP1 was a...
mitosis and proliferation regulator of cancer cells. As was expected, other studies and our findings revealed that SHCBP1 depletion impaired the proliferation of cancer cells in vitro, which might be caused by the abnormal cell division.

In summary, we found an upregulation of SHCBP1 in human EC tissues. Our findings further demonstrated that SHCBP1 expression was related to the clinical features of ESCC patients. We found that SHCBP1 depletion inhibited the proliferation and motility of ESCC cells via the TGFβ pathway, and suppressed tumor growth in mice. We, therefore, conclude that SHCBP1 could be a promising therapeutic target for the treatment of EC.

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