Overexpression SPRY2 Suppresses Esophageal Squamous Cell Carcinoma Progression Via Inactivation of ERK/AKT Signaling

La Sobreexpresión SPRY2 Suprime la Evolución del Carcinoma de Células Escamosas de Esófago Mediante la Inactivación de la Señalización ERK/AKT

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SUMMARY: Esophageal cancer is one of the most aggressive gastrointestinal cancers. Invasion and metastasis are the main causes of poor prognosis of esophageal cancer. SPRY2 has been reported to exert promoting effects in human cancers, which controls signal pathways including PI3K/AKT and MAPKs. However, the expression of SPRY2 in esophageal squamous cell carcinoma (ESCC) and its underlying mechanism remain unclear. In the present study, we aimed to investigate the detailed role of SPRY2 in the regulation of cell proliferation, invasion and ERK/AKT signaling pathway in ESCC. It was identified that the expression level of SPRY2 in ESCC was remarkably decreased compared with normal tissues, and it was related to clinicopathologic features and prognosis ESCC patients. The upregulation of SPRY2 expression notably inhibited the proliferation, migration and invasion of Eca-109 cells. In addition, the activity of ERK/AKT signaling was also suppressed by the SPRY2 upregulation in Eca-109 cells. Our study suggests that overexpression of SPRY2 suppress cancer cell proliferation and invasion of by through suppression of the ERK/AKT signaling pathways in ESCC. Therefore, SPRY2 may be a promising prognostic marker and therapeutic target for ESCC.

KEY WORDS: SPRY2; ERK; AKT; Esophageal squamous cell carcinoma(ESCC).

INTRODUCTION

Esophageal cancer (EC) is the eighth most common type of cancer worldwide and forms the sixth leading cause of cancer deaths, and is responsible for over 400,000 cases (Sung et al., 2021). Esophagus cancer affects more than 220,000 people in China each year and is the third most frequent digestive cancer (Jiang et al., 2021). The five-year survival rate of patients with advanced esophageal cancer was less than 20 percent, recurrence and metastasis are the main cause of poor prognosis (Chevallay et al., 2018). Therefore, revealing the molecular mechanism underlying ESCC development and progression may assist in finding more effective therapeutic strategies against ESCC.

SPRY protein was the first discovered in drosophila as an inhibitor for the stimulation of trachea branch development by FGF (fibroblast growth factor) (Locatelli et al., 2020). Subsequently, SPRY2 was identified to be an agonist of receptor tyrosine kinase (RTK) signal widely present in different morphogenetic process, such as trachea, lung, and other tissues (Kawazoe & Taniguchi, 2019). Most studies have shown that SPRY2 serves a suppressive role in different human cancer which plays an important role in inhibiting the cancer cells differentiation and migration (Patel et al., 2018; Locatelli et al., 2020; Hora et al., 2021). It has been demonstrated SPRY2 regulates PI3K/AKT and MAPK/ERK pathways by various growth factors, however, the expression and regulatory mechanism of SPRY2 in ESCC remains unclear. The aim of the current study was to investigate the role and underlying mechanism of SPRY2 in the regulation cancer cell proliferation, invasion and the involvement of ERK/AKT pathway in ESCC.

MATERIAL AND METHOD

Clinical samples and Ethics. A total of 80 formalin-fixed paraffin-embedded (FFPE) specimens from human ESCC

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with matched paracarcinomatous esophageal tissues were acquired from patients who underwent surgery at First Affiliated Hospital of Xinjiang Medical University during January 2017 and December 2020. Histological grade and TNM stage of tumor were performed according to the WHO classification and American Joint Committee on Cancer (AJCC) criteria, respectively. None of the patients received radiotherapy, chemotherapy and other treatments before surgery. The protocol and the informal consent was approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University and Written by each participant.

**Immunohistochemistry.** Immunohistochemical SP assay was used to detect SPRY2, p/t-ERK1/2 and p/t-AKT proteins expression in ESCC and normal adjacent tissues (NATs). SPRY2, p/t-ERK1/2 and p/t-AKT (Abcam plc, Cambridge, MA, USA), SP immunohistochemistry kit and DAB chromogenic agent (Zhong Shan Goldenbridge Biotechnology Co. Ltd, Beijing, China). Paraffin-embedded tissues were sectioned in 3 µm thickness, followed by conventional xylene dewaxing, gradient alcohol hydration, microwave for antigen repair, 3 % H₂O₂ 20 µl blocked with endogenous peroxidase inhibitor (Zhong Shan Goldenbridge Biotechnology Co. Ltd, Beijing, China) at room temperature for 30 min, and then incubated with antibodies against SPRY2, p/t-ERK1/2 and p/t-AKT overnight at 4 °C. Two experienced pathologists independently scored the staining patterns. Immunostaining scores were semi-quantitatively estimated according to staining intensity and distribution. The concentration of SPRY2, p/t-ERK1/2 and p/t-AKT antibodies were 1:150, 1:200 and 1:150, respectively. The next day, biotin-labeled antibody was added and incubated at 37 °C for 30 min. PBS buffer (pH7.4) and washed for 3 times (10 min) at each step. DAB color, hematoxylin contrast staining, conventional alcohol against concentration gradient dehydration, xylene transparent, neutral gum sealing. SPRY2 protein expression products were located in the cytoplasm, p/t-ERK1/2 and p/t-AKT proteins expression products were located in the cytoplasm or nucleus, and were positive when stained with brownish yellow granules. Each section randomly selected positive cells from10 high magnification field (HPEs×100/200/400), and the average number of positive cells per 100 cells was taken as the positive cell rate. Positive cells with SPRY2, p/t-ERK1/2 and p/t-AKT expression ≥25 % were considered as positive (+), while cells without staining or positive cell rate <25 % were considered as negative (-).

**Cell culture.** Human ESCC cell line Eca-109, Human cervical squamous cell carcinoma line Siha and Human colon carcinoma line SW620 cells were obtained from Beijing Bei-Na Biotechnology Research Institute (Beijing, China). Eca-109, SW620 and Siha cells were cultured with Dulbecco’s Modified Eagle Medium (DMEM) (Hyclone, USA), maintained in a 37 °C incubator containing 5 % CO₂ and routinely passaged at a density of 90 %. 10 % fetal bovine serum (FBS) (Gibco, New Zealand), 100 units/mL penicillin and streptomycin (Hyclone, USA) were added to the cell culture medium.

**Plasmid extraction and transformation.** Overexpressed (Catalog no.RC204864 - Pcmv6-Myc-DDK-Spyr2) was purchased from OriGene (USA). The plasmid was transfected into competent E.coli cells, and then monoclonal amplification was used. 200 µl DH-5a suspension was taken from the refrigerator at -80 °C and placed on ice for 30 min. Plasmid DNA solution (plasmid 2 ul, DD water 40 µl) was added and shaken, and left on ice for 30 min. And the plasmid was added into 500 µl LB liquid medium (without kanamycin), the mixture was then mixed and shaken at 37 °C for 1 h. 100 µl of bacterial liquid was prepared on solid LB plate containing kanamycin preheated to 37 °C. Plasmid extraction was performed according to the instructions, and the concentration of plasmid was detected by nucleic acid quantifier. The plasmid transfection was transduced using a Lipofectamine™2000 Transfection Reagent Kit (Catalog no. 11668019) on the basis of the instructions. Infection of the Eca-109 cell, Siha and SW620 cells lines were carried out in 6-well plates with serum-free DMEM. Cells were transfected with an unrelated control sequences in the negative control group, while cells were not transfected with any RNA in the blank control group. The transfection efficiency was observed under an inverted fluorescence microscope 24-48 h after transfection, and reached about 70 % for subsequent experiment.

**RNA isolation and Quantitative RT-PCR assay.** Total RNA was extracted from cell cultures grown to 80 % confluence using trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed into 2 mg cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) was used to carry out quantitative RT-PCR on an Applied Biosystems ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and annealing/elongation at 60 °C for 15 s. The -DDCq the 2-DDCq method analyzed the relative expression. The primer sequence is as follows: SPRY2 forward, 5'-CTTACTGTCCTCCCAGACCT-3' and reverse 5'-GGGGCTCGTGCAAGAAGAAT-3'. GAPDH forward, 5'-TATGAAATGTGCTGGTGA-3' and reverse 5'-CACAGGGGAGAATGACTT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference to normalize the gene expression levels. The DD cycle threshold method was used to calculate the mean relative change by three to five determinations relative quantitative determinations. Repeat for all reactions.
Western blot assay. Whole-cells were lysed by adding RIPA buffer (Thermo Fisher Scientific, Inc.). The BCA Protein Assay kit extracted and measured protein expression (Pierce; Thermo Fisher Scientific, Inc.). The protein was denatured by boiling for 5 min, and proteins (40 µg) was separated by 10 % SDS-PAGE and then transferred onto polyvinylidene difluoro-ride (PVDF) membrane (Thermo Fisher Scientific, Inc.). The Blots were incubated adding 5 % non-fat milk, and then with antibody targeting SPRY2 (1:300; rabbit anti-mouse.ab85670), or β-tubulin antibodies (dilution, 1:100; cat no. ab21058; Abcam, plc, Cambridge, MA, USA). Following the PVDF membranes with secondary antibody (dilution, 1:150; cat no. ab6721; Abcam) were incubated together for 1h at room temperature. Then enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific Corporation) was used to detect the experimental samples. The relative protein expression level was analyzed by Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

MTT assay. Eca-109 cell was at a density of 4000 cells/Well in 96-well plates incubated overnight. In the aftermath of the Eca-109 cells were attached, at 0h, 12 h, 24 h,48 h and 72 h, and then added into 200 µl MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(0.5 mg/ml). The Eca-109 cells were incubated for 4 h at 37 °C the MTT formazan precipitate was dissolved in 150 ml of DMSO (Sigma, USA) to solubilize the formazan crystals, in a shaker before test and the absorbance at 490 nm using a microplate autoreader (Bio-Rad, USA).

Invasion assay. Transwell chamber assay detected the Eca-109 cell invasion ability. After trypsin digestion and centrifugation, cell suspension was prepared. Apply diluted Matrigel adhesive to the small upper chamber surface of Transwell. 100 µl serum-free DMEM suspended Eca109-cells. Then cells seeded in the upper chamber. 600 µl of DMEM containing 10 % FBS was poured into in the chamber, leaved in the incubator for 24 h, and fixed by crystal violet staining. Wipe the cells with a wet cotton swab on the membrane. The number of migrated cells were counted by Nikon ECLIPSE TS 100 epifluorescence microscope and analyzed (×10) in ten random fields.

Migration assay. The wound healing assay analyzed the cell migration in 6-well plates. Briefly, 1ml of cells (1×105 cells/ml) was transferred into each well and incubated at 37 °C with 5 % CO₂. After appropriate cell attachment was achieved (24 h), assessed migration in different time points (0, 24, and 48 h), and captured images using the Nikon ECLIPSE TS100 epifluorescence microscope and the NIS Elements AR 3.1 software.

Statistical analysis. SPSS software package (version 19.0; SPSS, Inc. Chicago, IL, USA) and Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA) were used for the analysis of experimental data. The data are signified as the mean±standard deviation (SD) following at least three independent experiments. The Mann–Whitney test was used to test continuous variables for differences in SPRY2 immunohistochemistry scores between ESCC and normal tissues. The relationships between SPRY2 expression and clinicopathological characteristics were tested by the Chi-square test or Fisher’s exact test, as appropriate. Results were considered statistically significant when P<0.05.

RESULTS

Expressions of SPRY2, t/p-ERK1/2 and t/p-AKT in ESCC. Compared to normal tissues, the expression of SPRY2 protein was significantly reduced (55 %) in ESCC tissues, the difference was statistically significant (P<0.05) (Fig. 1a and b). The expression levels of SPRY2 in the poorly differentiated group, the infiltrating muscular layer group and the lymph node metastasis group were lower than those

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**Fig. 1.** Immunohistochemical staining of SPRY2, t-ERK1/2, p-ERK1/2, t-AKT and p-AKT proteins in normal esophageal mucosa and ESCC. a) normal esophageal mucosa (×400), b) ESCC tissue (×400).
in the high differentiated group, the infiltrating mucosal layer group and the non-metastasis group. While the positive expression rates of p-ERK1/2 and p-AKT in ESCC tissues were 91.25% and 87.5%, respectively, which were obviously higher than those in normal tissues (Fig. 1a and b). The expression levels of p-ERK1/2 and p-AKT in the poorly differentiated and the lymph node metastasis group were higher than those in the differentiated, non-metastasis group. Therefore, the expression of SPRY2 protein was increased and the expression of p-ERK1/2, p-AKT proteins were decreased in ESCC, which were closely related to clinicopathological features (Table I).

SPRY2 expressions in different cancer cell lines. We used Western blot and RT-PCR to examine expression of SPRY2 in Human ESCC line Eca-109, Human cervical squamous cell carcinoma line Siha and Human colon carcinoma line SW620. The data indicated that expression levels of SPRY2 were different in Eca-109, Siha and SW620 cell lines. However, data analysis showed that the difference was not statistically significant (P<0.05) (Fig. 2a and b).

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Spry2 Positive (%)</th>
<th>X2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>80</td>
<td>85</td>
<td>1.13</td>
<td>0.25</td>
</tr>
<tr>
<td>Tumor</td>
<td>80</td>
<td>55</td>
<td>5.63</td>
<td>0.02</td>
</tr>
<tr>
<td>Differentiation</td>
<td>48</td>
<td>2.02</td>
<td>41.77</td>
<td>0.04</td>
</tr>
<tr>
<td>High-moderate</td>
<td>32</td>
<td>2.00</td>
<td>93.75</td>
<td>0.67</td>
</tr>
<tr>
<td>Poor</td>
<td>10</td>
<td>2.50</td>
<td>5.67</td>
<td>0.01</td>
</tr>
<tr>
<td>Depth</td>
<td>70</td>
<td>90</td>
<td>1.96</td>
<td>0.10</td>
</tr>
<tr>
<td>Mucosa</td>
<td>33</td>
<td>33</td>
<td>3.93</td>
<td>0.01</td>
</tr>
<tr>
<td>LN metastasis</td>
<td>Positive</td>
<td>52.77</td>
<td>5.27</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Effect of SPRY2 overexpression on ERK/AKT signaling pathway in Eca-109 cell

Expression of SPRY2 mRNA and protein after transfection. Following transfection with SPRY2 agonists, the expression of SPRY2 mRNA in the agonist group was significantly higher than that in the two control groups (P<0.01) (Fig. 3a). Meanwhile, Western blot results showed that SPRY2 protein levels were significantly increased in the SPRY2 agonist group compared with the other two groups (P<0.01) (Fig. 3b).

The effect of overexpression of SPRY2 on the expression of ERK/AKT proteins in Eca-109 cells. Western blot results showed that compared with NC group and CV group, the expression level of SPRY2 protein in SPRY2 agonist group was significantly increased (P<0.001), while the protein expression levels of p-ERK1/2 and p-AKT were significantly decreased (P<0.01) (Fig. 4a).
findings suggest that t-ERK1/2 and t-AKT expression was altered, but the difference was not statistically significant (P<0.05) Fig. 4b.

**SPRY2 decreases the proliferation and migration in Eca-109 cells.**

**Overexpression of SPRY2 inhibited cell proliferation in Eca-109 cells.** The effects of SPRY2 overexpression on the proliferation and migration of ESCC were detected by MTT assay. It was detected that cell proliferation was significantly inhibited in the SPRY2 agonist group compared with the NC group and the CV group, the ability of Eca-109 cells to proliferate was obviously decreased (Fig. 5).

**Overexpression of SPRY2 inhibited cell migration in Eca-109 cells.** The result of a wound healing assay demonstrated that the inhibition rate of SPRY2 on wound healing at 24 h and 48 h was 25.77 % and 64.47 % (P<0.01) (Table II). Over time, compared with the NC group and the CV group, the SPRY2 agonist group showed decreased migrate capacity. A significant reduction in the ability of cells to invasion was also observed.
Table II. Overexpression of Spry2 inhibited cell migration in Eca109 cells (mean±SD).

<table>
<thead>
<tr>
<th>Time</th>
<th>NC</th>
<th>VC</th>
<th>Spry2</th>
<th>Value P</th>
<th>Inhibition rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>20.3±0.58</td>
<td>19.3±0.58</td>
<td>19.5±0.50</td>
<td>0.789</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>16.1±0.29</td>
<td>17.3±0.58</td>
<td>20.3±1.53</td>
<td>0.009</td>
<td>25.77 %</td>
</tr>
<tr>
<td>48 h</td>
<td>12.6±0.58</td>
<td>14.1±0.76</td>
<td>20.8±2.02</td>
<td>&lt;0.01</td>
<td>64.47 %</td>
</tr>
</tbody>
</table>

DISCUSSION

Esophageal cancer is a malignant digestive tumor with poor prognosis, the main reason is that most patients are in the advanced stage when they are diagnosed, overall 5-year survival rate is only 15%-25%. Therefore, identifying susceptible genes and biomarkers could provide implications for early diagnosis, potential therapeutic strategies while improve their survival rates.

Sprouty (SPRY) mammalian proteins are composed of four evolutionarily conserved family members (SPRY 1-4) that exhibit tissue-specific expression patterns, apart from the most conserved sprouty isoform SPRY2 that is widely expressed (Sripada et al., 2021). SPRY2 is deemed most important in many adult tissues, which is most well-characterized role in regulating extracellular signal–regulated kinase (ERK) activity. In the recent years, SPRY2 has been found to play crucial role in various cancers. Growing evidence has mainly implied that Sprouty2 (SPRY2) as a tumor suppressor in many solid tumors, which can promote tumor cells apoptosis and inhibit their proliferation, invasion and migration ability (Sun et al., 2020; Schumacher et al., 2021). In many instances, downregulation of SPRY2 has been observed in prostate (Samadaian et al., 2018), liver (Kovi et al., 2019), lung (Yawut et al., 2020), and pancreatic cancer (Li et al., 2022), and also has been found to influence EMT process in gastric (Li et al., 2021). However, this finding is not uniformly observed across all cancer cell. For example, Walsh et al. (2015) found that SPRY2 surprisingly acted as a driver of GBM cell proliferation, which resistance to inhibition of RTK and promote GBM survival. It has also been reported that SPRY2 controls pathways including PI3K/AKT and MAPK/ERK cascades, which is indispensable for mucosal epithelial homeostasis and directs intestinal epithelial cell development and differentiation (He et al., 2018). To date, the expression SPRY2 in ESCC and its underlying molecular mechanisms remain elusive.

On the basis of our study, the result indicated that the positive expression rate of SPRY2 protein in ESCC tissues were higher than those in normal esophageal tissues. Besides, the decreased expression of SPRY2 in ESCC specimens is related to the depth of tumor invasion, lymph node metastasis and degree of differentiation. Furthermore, the positive expression rates of p-ERK1/2 and p-AKT proteins in ESCC tissues were higher than those in normal esophageal tissues, which was also associated with clinical pathologic features of ESCC. The experimental results in this part of the study are basically consistent with the relevant reports, indicating that SPRY2, p-ERK1/2 and p-AKT have certain value in the diagnosis of ESCC and the judgment of malignant degree and prognosis.

The second part of our study mainly focused on analyzed the effect of SPRY2 expression level on the proliferation and invasion of esophageal cancer cells at the cell level in vitro. Our results showed that with the overexpression of SPRY2, the proliferation, migration, invasion and wound healing process of Eca-109 cells were inhibited. Samadaian et al. (2018) also revealed that SPRY2 inhibited cell proliferation and migration in prostate cancer cells. Li et al. (2022) reported that SPRY2 was downregulated in pancreatic ductal adenocarcinoma tissues compared with adjacent normal tissues, and that SPRY2 could induces EMT and promotes metastasis by activation of the b-catenin/TCF4 pathway. Therefore, our findings suggest that SPRY2 mainly acts as a tumor suppressor, which play important role in cancer cell proliferation and migration.

Furthermore SPRY2 reveals a stronger inhibitory effect on ERK activation, which binds the adaptor protein GRB2 and interacts with Raf downstream of Ras. Thus, SPRY2 interferes with the ERK pathway upstream and downstream of Ras. Atif et al. (2022) demonstrated that SPRY2 could promotes Akt-induced HCC development through the activation of Ras/Raf/MEK/ERK pathway and cooperates with activated b-catenin to induce HCC in Huh7 cell lines. Evidence was provided for an inhibitory role of SPRY2 on AKT and p38 signaling by enhancing the activity of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Park et al., 2018). We also observed that overexpression of SPRY2 with a concomitant inhibition of the ERK/AKT pathway, and the invasion, migration and proliferation of esophageal cancer cells were reduced, suggesting that SPRY2 regulates the development of esophageal cancer by regulating the ERK/AKT target. Xiao et al. (2018) also confirmed miR-330-5p via targeting SPRY2 to activate MAPK/ERK signaling in promoting HCC progression. These results suggested that overexpression of SPRY2 suppressed proliferation and...
invasion through functional inhibition of the ERK/AKT pathway in ESCC cells.

CONCLUSIONS

Our study provides compelling evidence that SPRY2 expression is obviously reduced in ESCC and its low expression is statistically correlated with clinicopathologic characteristics and poor prognosis in patients with ESCC. Overexpression of SPRY2 not only inhibited the migration and invasion of ESCC cells, but also inhibited the functional activation of the ERK/AKT pathway. SPRY2 may represent a novel prognostic biomarker as well as a potential therapeutic target for ESCC therapy.

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