

# Substance P is overexpressed in cervical squamous cell carcinoma and promoted proliferation and invasion of cervical cancer cells *in vitro*

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## ABSTRACT

This study aimed to investigate the expression and function of substance P in cervical squamous cell carcinoma. Cancer tissues and adjacent tissues of 20 patients with cervical squamous cell carcinoma in our hospital were collected. The expression of substance P was detected by immunohistochemistry and Western blot analysis. Cervical squamous cell carcinoma line SiHa was treated with different concentrations of substance P. The proliferation of SiHa cells was detected by EdU assay, and the invasion ability of SiHa cells was detected by transwell assay. The phosphorylation of ERK1/2 and the expression of MMP9 were detected by Western blot analysis. The results showed that substance P was expressed in the cytoplasm and some cell membranes of cervical squamous cell carcinoma cells. The expression of substance P in cervical cancer tissues was significantly higher than that in the adjacent tissues. Compared with the control group, substance P significantly promoted the proliferation and invasion of SiHa cells in a concentration dependent manner and activated the phosphorylation of ERK1/2 and upregulated the expression of MMP9 in SiHa cells. In conclusion, substance P is highly expressed in cervical squamous cell carcinoma and can promote cervical cancer cell proliferation and invasion. The mechanism is related to the activation of ERK1/2 pathway to upregulate MMP9.

**Key words:** substance P; cervical cancer; proliferation; invasion; ERK; MMP9.

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**Ethics approval:** this study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (approval no. 20190312). All patients signed written informed consent.

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## Introduction

Cervical cancer is the fourth most common malignant tumor in women worldwide, and persistent infection with high-risk human papillomavirus is the main cause of this disease.<sup>1</sup> Although comprehensive treatment options such as surgery, radiotherapy and chemotherapy can improve the efficacy of cervical cancer, the prognosis of patients with advanced stage or relapse of cervical cancer is poor and the efficacy is unsatisfactory.<sup>2</sup> Therefore, recent studies have focused on the pathogenesis of cervical cancer to develop effective treatment methods at molecular level.

Substance P (SP) is a type of neurotransmitter that is widely distributed in various tissues including the nervous system. SP is involved in mediating a variety of biological functions, such as pain transmission, contraction of gastrointestinal smooth muscle, inflammation and the regulation of immune response. In recent years, accumulating studies have shown that SP is highly expressed in different tumor tissues and cells.<sup>3-5</sup> In addition, SP can reduce the apoptosis of tumor cells, promote the proliferation of tumor cells, promote angiogenesis in the tumor microenvironment, and enhance the invasion and metastasis of tumor cells.<sup>6,7</sup> However, the expression and function of SP in cervical cancer remain largely unknown. In this study, we aimed to detect the expression of SP in cervical squamous cell carcinoma and investigate the function of SP in cervical squamous cell carcinoma cells.

## Materials and Methods

### Clinical samples

The clinical samples were from 20 patients with cervical squamous cell carcinoma without any preoperative treatment in the Fourth Hospital of Hebei Medical University from December 2019 to June 2020. The patients are aged 28-67 years. According to FIGO staging, there were 12 cases of stage I-II and 8 cases of stage III-IV. All patients signed written informed consent. This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Approval No. 20190312).

### Immunohistochemistry

The immunohistochemical streptomyacin avidin-peroxidase method was used to detect the expression of SP in clinical samples. The cervical cancer tissues and adjacent cervical tissues were fixed in formalin and embedded in paraffin, then cut into 5  $\mu$ m thin sections. Antigen retrieval was performed by boiling the sections in 10 mM citrate buffer (pH 6.0) for 10 min. The sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidases. Next, the sections were incubated with SP antibody (1:800 dilution; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C, washed with phosphate-buffered saline (PBS), and then incubated with SP-9001 kit (Zhong Shan Golden Bridge, Beijing, China) to visualize the staining. PBS instead of the primary antibody was used as the negative control. According to the staining intensity, no color, light yellow, brownish yellow and brown were judged as 0, 1, 2 and 3 points, respectively. According to the percentage of stained cells, <1%, 1-10%, 10-50%, 50-80% and >80% were judged as 0, 1, 2, 3 and 4 points, respectively. Allred scoring system was used based on the sum of the two scores, 0 was negative (-), 1-2 was weak positive (+), 3-5 is medium positive (++), and 6-7 was strong positive (+++).<sup>8</sup>

## Cells and treatment

Human cervical cancer cell line SiHa was purchased from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, GIBCO, Waltham, MA, USA) at 37°C with 5% CO<sub>2</sub>. SP was purchased from Sigma-Aldrich and dissolved in distilled water. Cells were treated with SP at the concentration of 0.1  $\mu$ M, 1  $\mu$ M or 10  $\mu$ M for 24 h.

### EdU assay

Cell proliferation was evaluated using EdU cell proliferation assay kit (C10812-1, Ribobio, Guangzhou, China) following the manufacturer's protocols. Briefly, SiHa cells were seeded in 96-well plates and cultured for 48 h with the treatment with different concentrations of SP. Next, the cells were washed and incubated with 10  $\mu$ M EdU for 2 h at 37°C. The nuclei were stained with 5  $\mu$ g/mL Hoechst 33342 (Sigma-Aldrich) for 30 min at room temperature in the dark. The cells that incorporated EdU were observed under Nikon Eclipse E600 fluorescence microscope at 200x magnification, and the percentages of cells positive for EdU staining were calculated from three randomly selected microscope fields.

### Transwell assay

SiHa cells in the logarithmic growth stage were collected and suspended in RPMI 1640 medium at the concentration of 2 $\times$ 10<sup>6</sup>/mL. Next, 500  $\mu$ L of cell suspension was mixed with different concentrations of SP and added to the upper chamber of transwell (BD Biosciences, Franklin Lakes, NJ, USA), while the lower chambers were filled with 500  $\mu$ L of RPMI 1640 medium supplemented with 10% FBS. After 24 h incubation at 37°C, the cells on the upper surface of the filters were wiped with a cotton swab and the cells on the underside of the filters were fixed, stained with crystal violet, and the number of cells in 5 fields of view was counted under light microscope (Olympus IX71, original magnification 100x).

### Western blot analysis

The total protein was extracted from tissues or cells using RIPA buffer and the concentration was quantitated by BCA method. Equal amounts of proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% non-fat milk and then incubated with primary antibodies for ERK1/2, p-ERK1/2, MMP9 and  $\beta$ -actin (all from Abcam, Cambridge, UK). The membranes were washed and incubated with secondary antibodies (Abcam), and detected by chemiluminescence. Densitometry of the bands was analyzed by using Image-J software.

### Statistical analysis

SPSS 19.0 statistical software was used for data processing. The data were expressed in mean  $\pm$  SD, and analyzed by one-way ANOVA. A p-value <0.05 was considered statistically significant.

## Results

### Expression of SP in cervical squamous cell carcinoma tissues

SP was expressed in the cytoplasm and some cell membranes of cervical squamous carcinoma cells (Figure 1A), and the positive expression rate in tissues was 65.00%. SP was rarely expressed in

the adjacent tissues and was occasionally expressed in the cytoplasm of cells (Figure 1B), with a positive expression rate of 20.00%. Western blot analysis showed that protein level of SP in cervical squamous cell carcinoma tissues was higher than that in the adjacent tissues (Figure 1C). Densitometry analysis showed that the difference in SP protein level between cervical squamous cell carcinoma tissues and adjacent tissues was statistically significant (Figure 1D).

### SP promoted the proliferation of SiHa cells

To reveal the function of SP in cervical squamous carcinoma, we used the cervical squamous carcinoma cell line SiHa as the model. First, we examined the effect of SP on the proliferation of SiHa cells. EdU assay showed that the number of SiHa cells positively stained with EdU increased with higher concentrations of SP (Figure 2A). Statistical analysis showed that SP significantly promoted the proliferation of SiHa cells in a dose-dependent manner (Figure 2B).

### SP promoted the invasion of SiHa cells

Next, we examined the effect of SP on the invasion of SiHa cells. Transwell assay showed that the number of SiHa cells that invaded the filters increased with higher concentrations of SP (Figure 3A). Statistical analysis showed that SP significantly promoted the invasion of SiHa cells in a dose-dependent manner (Figure 3B).

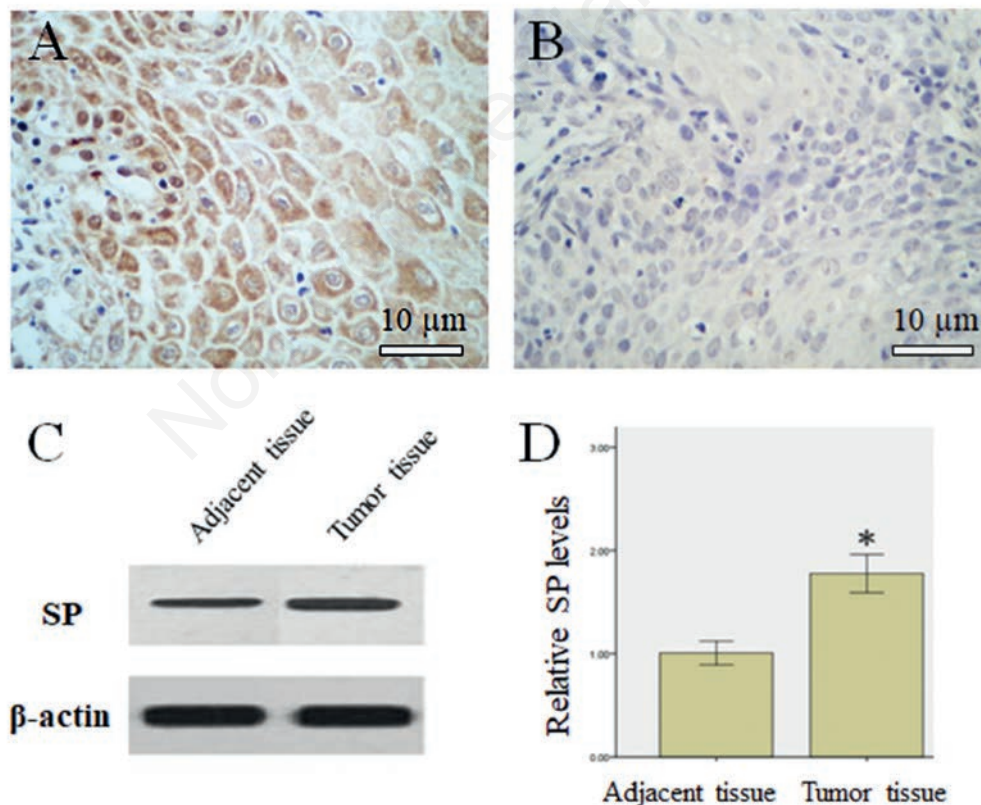
### SP promoted the activation of ERK1/2 and the upregulation of MMP9 in SiHa cells

To elucidate the mechanism by which SP promoted the invasion of SiHa cells, we examined the signaling pathway involved in cell invasion. Western blot analysis showed that protein levels of MMP9 and p-ERK1/2 were higher in SiHa cells treated with higher concentrations of SP (Figure 4A). Densitometric analysis showed that SP significantly increased MMP9 and p-ERK1/2 protein levels in a dose-dependent manner (Figure 4 B,C).

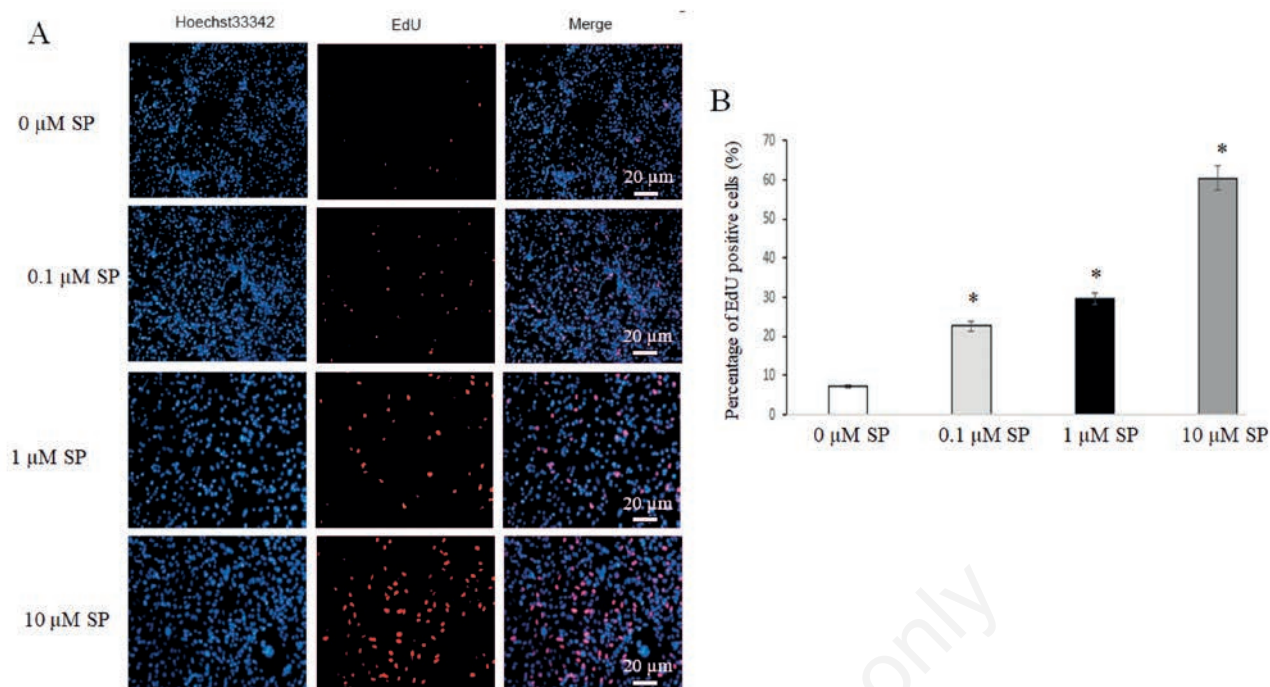
### Discussion

In this study, immunohistochemistry and Western blot analysis confirmed that the expression of SP in cervical squamous cell carcinoma tissues was significantly higher than that in the adjacent normal tissues, indicating that SP is involved in the occurrence and development of cervical squamous cell carcinoma. Furthermore, using SiHa cells as a cell model we found that SP significantly promoted the proliferation and invasion of cervical cancer cells, suggesting that SP is a new target for the treatment of cervical cancer.

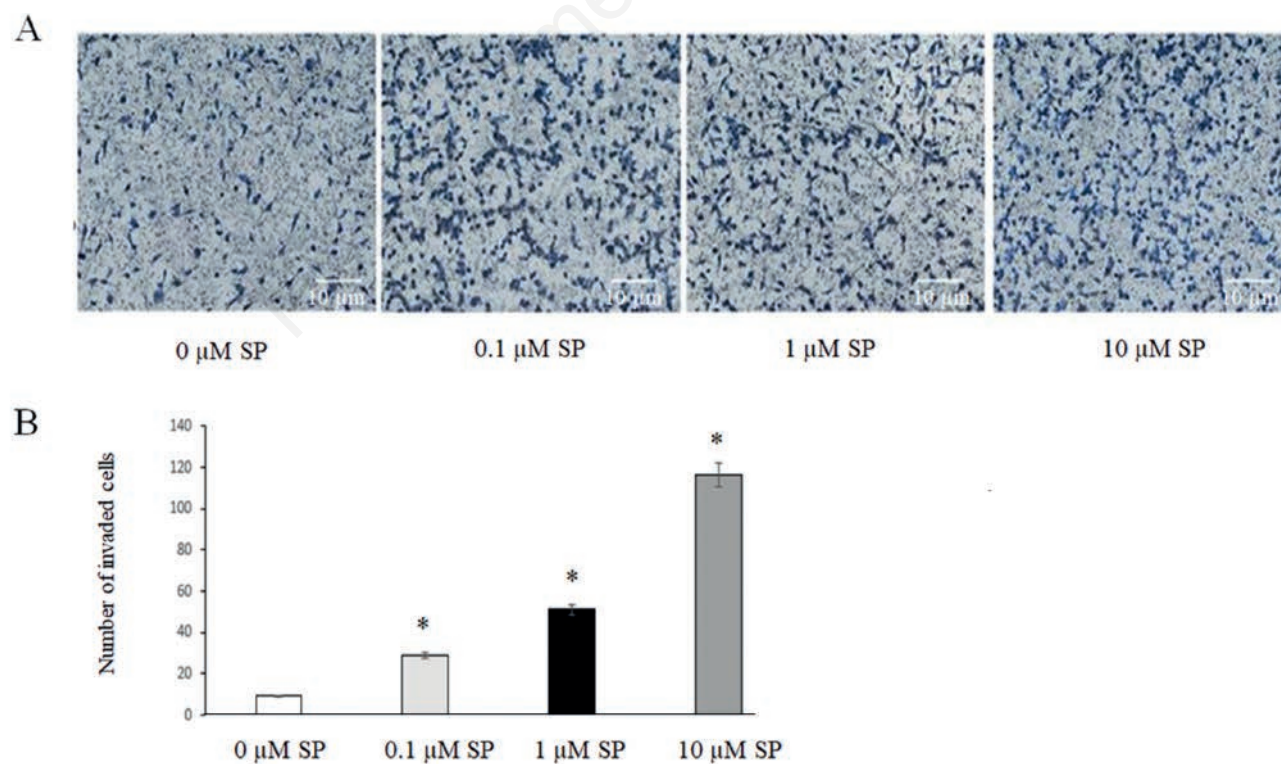
Cervical cancer remains a big medical problem for women.<sup>9</sup> Recent studies have shown that ERK signaling is activated in cervical cancer.<sup>10-12</sup> In fact, Lu *et al.* pointed out that about one third of all types of tumors were related to ERK1/2 activation.<sup>13</sup>



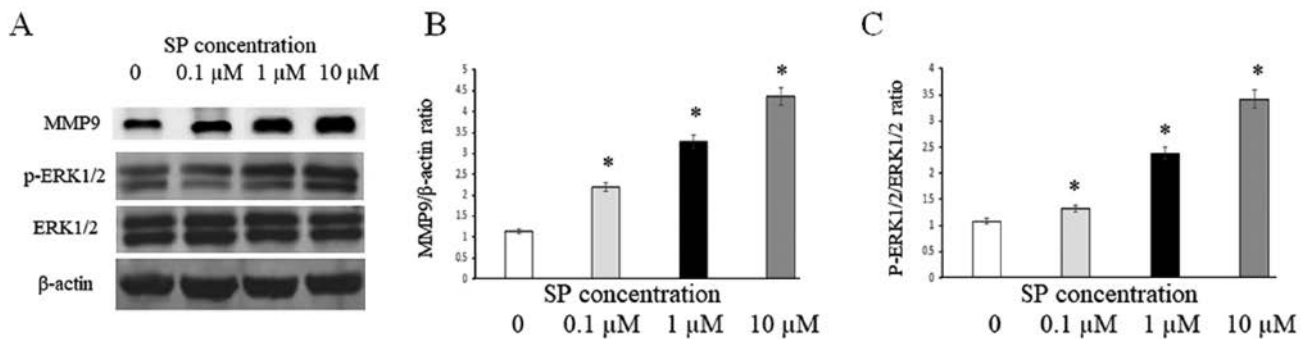
**Figure 1.** The expression of SP in cervical squamous cell carcinoma tissues. **A)** SP was expressed in the cytoplasm and some cell membranes of cervical squamous carcinoma cells. **B)** SP was rarely expressed in the adjacent tissues and was occasionally expressed in the cytoplasm of cells. **C)** Western blot analysis of SP protein levels in cervical squamous cell carcinoma and adjacent tissues. **D)** Densitometric analysis of SP protein levels in cervical squamous cell carcinoma and adjacent tissues. Data were expressed as mean  $\pm$  SD (n=3). \* $p < 0.05$  compared to control.



**Figure 2.** TSP promoted the proliferation of SiHa cells. **A)** EdU assay of SiHa cells treated with different concentrations of SP. **B)** Statistical analysis of SiHa cells positively stained with EdU. Data were expressed as the mean  $\pm$  SD (n=3). \*p<0.05 compared to control cells.



**Figure 3.** SP promoted the invasion of SiHa cells. **A)** Transwell assay of SiHa cells treated with different concentrations of SP. **B)** Statistical analysis of SiHa cells that invaded the filters of transwell. Data were expressed as the mean  $\pm$  SD (n=3). \*p<0.05 compared to control cells.



**Figure 4.** SP activated ERK1/2 and upregulated MMP9 in SiHa cells. **A)** Western blot analysis of MMP9, p-ERK1/2 and ERK1/2 protein levels in SiHa cells treated with different concentrations of SP; actin was loading control. **B)** Densitometry analysis of MMP9/actin ratio in SiHa cells treated with different concentrations of SP. **C)** Densitometry analysis of p-ERK /ERK ratio in SiHa cells treated with different concentrations of SP. Data were expressed as mean  $\pm$  SD (n=3). \*p<0.05 compared to control cells.

Activated ERK1/2 can enter the nucleus and activate nuclear transcription factors to promote cell proliferation and invasion.<sup>14,15</sup> In addition, ERK1/2 is associated with the regulation of apoptosis, and activated ERK1/2 protects cells from apoptosis by increasing or decreasing the levels of anti-apoptotic and pro-apoptotic proteins.<sup>16,17</sup> Zhao *et al.* showed that activated ERK1/2 could induce cell cycle arrest in the G<sub>2</sub>/M phase, thereby inhibiting apoptosis.<sup>18</sup> Kurland *et al.* reported that ERK1/2 could activate NF $\gamma$ B1 dimer, which was an important regulator of anti-apoptotic proteins such as Bcl-2 family.<sup>19</sup> In this study we found that SP promoted the proliferation of cervical cancer cells, but the mechanism remains unclear. Further studies are needed to examine the link between SP/ERK signaling and anti-apoptotic proteins in cervical cancer cells.

MAPK-ERK signaling plays an important role in the regulation of cancer cell invasion.<sup>20,21</sup> It is known that MMPs family could degrade extracellular matrix and promote tumor metastasis. Interestingly, the activation of MAPK-ERK signaling leads to the upregulation of MMPs and the increase in cancer cell invasion ability.<sup>22</sup> Furthermore, the activation of ERK1/2 signaling is associated with tumor angiogenesis.<sup>23,24</sup> Activated ERK stimulated the expression of VEGF and promoted the binding of VEGF-VEGF-2R, which contributes to tumor angiogenesis. Therefore, the activation of ERK1/2 can promote cancer metastasis. In this study we found that SP significantly increased the levels of p-ERK1/2 and MMP9 in cervical cancer cells, indicating that SP can promote ERK phosphorylation and transmit extracellular signals to the nucleus to upregulate the expression of MMP9, which in turn promotes the invasion of tumor cells. In future studies we will examine the role of SP in cervical cancer metastasis using *in vivo* model.

This study has several limitations. First, the number of patients is small. Second, the involvement of ERK1/2 pathway in mediating the effects of SP on cervical cancer cells should be confirmed by employing the inhibitors of ERK1/2 pathway. Third, the potential interaction of SP with interleukins in tumorigenesis should be further investigated.<sup>25</sup>

In summary, in this study we found that SP was highly expressed in cervical squamous cell carcinoma and promoted the proliferation and invasion of SiHa cervical cancer cells *in vitro*. The mechanism is related to the activation of ERK1/2 pathway to upregulate MMP9. These findings suggest the oncogenic role of SP in cervical squamous cell carcinoma and indicate that SP might be a potential target for the treatment of this tumor.

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