RESEARCH ARTICLE

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Syntaxin 6 Enhances the Progression of Epithelial Ovarian Cancer by Promoting Cancer Cell Proliferation

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Abstract

Objectives: The aim of this study is to evaluate the expression of syntaxin 6 (STX6) in epithelial ovarian cancer (EOC) and assess the effects of STX6 on the prognosis of patient. **Methods:** Using information from the Kaplan-Meier Plotter database, the effects of *STX6* expression on overall survival (OS) and progression-free survival (PFS) in ovarian cancer patients were examined. The clinical information of 147 patients with epithelial ovarian cancer was evaluated, and immunohistochemical staining was used to identify *STX6* expression in postoperative tumor specimens, and the affection of *STX6* expression on patient prognosis was assessed. In addition, the expression of STX6 in tumor tissue, peritoneal metastases (PM) derived from 13 patients with epithelial ovarian cancer and 6 normal ovarian specimens was detected by PCR and Western blot. In order to investigate how STX6 affects the proliferation of tumor cells, STX6 was also over expressed and knock down in ovarian cancer cell lines. Then colony formation assay was used to explore the effect of STX6 regulating on cell proliferation. **Results:** Kaplan-Meier Plotter enrollment data analysis revealed that patients with overexpressed STX6 had substantially worse OS and PFS than individuals with low *STX6* expression. Retrospective study revealed a significant ($P<0.05$) correlation between the *STX6* expression and tumor classifications, tumor stage, peritoneal carcinomatosis index (PCI), and PFS survival of patients. Western blot and PCR findings for fresh samples showed that STX6 was overexpressed in both primary lesions and PM nodules of OC. SKOV3 cell proliferation was shown to be dramatically reduced by STX6 knockdown and promoted by *STX6* overexpression, according to the in vitro experiments. **Conclusion:** STX6 may increase the progression of epithelial OC by encouraging the proliferation of cancer cells, indicating that STX6 was a viable therapeutic target of epithelial OC.

Keywords: Epithelial ovarian cancer- Syntaxin 6- Proliferation- Prognosis

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Introduction

As a common malignancy of the female reproductive system, ovarian cancer (OC) has the highest death rate of all gynecological malignancies (Younes and Zayed, 2019), posed a "silent killer" to the health of women. The pathological types of OC mainly including epithelial tumors, germ cell tumors, and sex cord stromal tumors. Among them, epithelial OC accounts for more than 95% of ovarian malignancies, and was more common in postmenopausal women (Lheureux et al., 2019). More than 70% of newly diagnosed ovarian cancer cases were classified as stage III or IV (Stewart et al., 2019). Although surgery-centered comprehensive treatment has been widely used in patients in recent years, and the treatment effect of OC patients has been continuously improved, the 5-year overall survival rate of patients was still only about 45% (Giampaolino et al., 2019; Morand et al., 2021). In addition, a growing percentage of patients are acquiring medication resistance to platinum-based treatment. Therefore, it was critical to identify new pathways contributing to the development of OC and find efficient substitute therapies. N-ethylmaleimide -sensitive factor attachment protein receptor (SNARE) is a kind of membrane protein involved in vesicle transport. During vesicle transport, SNARE complex can anchor the vesicle and target membrane together, thus promoting vesicle and target membrane fusion (Zorec, 2018). As a member of SNARE protein family, syntaxin6 (STX6) mainly exists on the membrane of trans Golgi apparatus, and regulates various intracellular transport activities, such as endocytosis, circulation, anterograde and retrograde transport (Dingjan et al., 2018; Wendler and Tooze, 2001). Recent studies have shown that intracellular vesicle transport, in particular the soluble SNARE complex, was involved in the process of tumor genesis, progression, and

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metastasis (Gorshtein et al., 2021; Meng and Wang, 2015). Among them, STX6, one of the important sensitive factors in SNARE, has been found to be expressed in brain, lung, kidney and other tissues (Bock et al., 1996; Peak et al., 2019). As a vesicle transporter, the important role of STX6 in intracellular protein transport and membrane structure alteration has been identified in previous studies (Zhang et al., 2022). Recent studies have found that STX6 was overexpressed in a variety of tumors, including esophagus, cervix, breast, liver, bladder, and prostate (Riggs et al., 2012) . And the highly expressed STX6 was closely related to pathological parameters such as tumor size, lymphatic metastasis, tumor stage, and tissue differentiation. However, the expression of STX6 in epithelial OC and its effects remain unclear.

In this study, we analyzed the correlation between *STX6* gene expression and patient prognosis in the Kaplan-Meier Plotter database. Then, the expression of STX6 in tumors of 147 patients with epithelial ovarian cancer was further detected, and the relationship between the expression level of STX6 and the clinicopathological parameters of patients was analyzed. Moreover, we further examined *STX6* expression in fresh samples from normal ovaries, tumors, and peritoneal metastases. Finally, after using siRNA to knock out the expression of STX6 in ovarian cancer cells, the effect of STX6 on the proliferation of tumor cells was discussed. Our data suggest that STX6 is involved in the progression of epithelial ovarian cancer by promoting tumor cell proliferation, and ultimately affects patient outcomes. This study explores the expression of STX6 in epithelial ovarian cancer and its effect on the prognosis of patients, which provides ideas for studying the therapeutic targets of ovarian cancer.

Materials and Methods

Database

In this study, data of 655 patients with ovarian cancer were obtained from the KaplanMeier Plotter database (Chen et al., 2021) (http://kmplot.com/). The patient population was screened in the database using the following keywords: (1) Cancer: Ovarian Cancer: (2) Gene: STX6; (3) Survival: overall survival or progression-free survival. STX6 expresses cutoff values: all possible cutoffs between the upper and lower quartiles, and automatically selects the best performing threshold as the cutoff value. The correlation of STX6 and prognostic values including PFS (progression-free survival) and OS (overall survival) were evaluated. Hazard ratios (HRs) with 95% confidence intervals and logrank P-value were determined. The survival curve was plotted by Kaplan-Meier, the Log-rank test was used to compare the difference between the cohorts of high expression and low expression, $P<0.05$ was determined as statistically significant.

Patients and tissue specimens

Paraffin embedded tumor tissues

Clinical data of 147 patients with epithelial ovarian cancer who were treated in the Affiliated Hospital of North Sichuan Medical College from January 2016 to September 2021 was reviewed and analyzed. Immunohistochemistry stain was used to detect *STX6* expression in the paraffin embedded tumor tissues of these 147 patients, and the correlation between *STX6* expression and clinical characteristics and prognosis was analyzed.

Fresh tissue specimens

Fresh tumor specimens of primary lesions and peritoneal metastases from 13 epithelial ovarian cancer patients from the Affiliated Hospital of North Sichuan Medical College were collected. The implementation plan of this study was approved by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College. None of the patients was treated with such preoperative therapies as radiation, chemotherapy, or immunotherapy. normal ovarian specimens from 6 patients with benign diseases (uterine fibroids and adenomyosis) were selected as controls. *STX6* expression in these fresh specimens was detected by PCR and Western Blot.

Cell culture

Human ovarian cancer cell line SKOV3 was purchased from Procell Life Science (Guangzhou, China) and cultured in DMEM medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (Gibco; USA) and 1% penicillin/streptomycin (GIBCO). Cells are cultured in a humidited 5% CO_2 incubator at 37°C.

Real-time PCR

The relative syntaxin 6 mRNA transcripts were measured by quantitative real-time RT-PCR. First, the total RNA of fresh samples was extracted by using the total RNA Extraction Reagent (Trizol, R0016, Beyotime) according to the manufacturer's instruction. Then 1 μg of total RNA was reverse-transcribed in a 20 μL reaction mixture using Transcriptor cDNA Synth. Kit2 (Roche). An SYBR Green assay was performed to analyze the expression level of syntaxin 6 mRNA, and the expression level of GADPH was used as the internal reference. Quantitative RT-PCR (QPCR) was performed using the SYBR PCR master mix in the Applied Biosystems 7500 Real-Time PCR Systems (Applied Biosystems). PCR was carried out in 20μL reaction total. The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 1 min. The relative level of miRNA expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. Each assay was performed in triplicate.

Western Blot

Tissue and cell samples were lysed on ice by lysate (ST506, Beyotime) containing 100 μg/mL phenylmethylsulfonyl fluoride (PMSF) (P0013, Beyotime) for 30 min. Then the lysates were collected and centrifuged at 10,000 rpm/min for 30 min, and the supernatants were collected. The samples of cell lysates or exosomes were quantified using the bicinchoninic acid assay (BCA) protein assay kit (P0012S, Beyotime). Samples were diluted with 2× SDS loading buffer and boiled to denaturation. The protein samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad protein analyzer (Bio-Rad) and transferred

to a polyvinylidene fluoride filter (PVDF) membrane (Millipore). Then the PVDF membrane was blocking with TBST containing 5% nonfat dried milk for 2 h at room temperature. Then the membranes were incubated with rabbit anti-human anti-STX6 antibody (1:1,000; Affinity Biosciences) or rabbit anti-human anti-GAPDH (1:500; Affinity Biosciences) at 4°C overnight. After washed by TBST buffer 3 times, membranes were incubated with HRP Goat Anti-Rabbit secondary antibody (1:5,000, Affinity Biosciences) for 2 h. After washed with TBST 3 times, an enhanced chemiluminescence reagent (Thermo Fisher Scientific) was added on the membranes, and the protein band were imaged using a FluorChem Q system (CA).

Immunohistochemical staining and scoring system

Paraffin-embedded tissue was dewaxed after sectioning and then blocked by incubated with goat serum at room temperature for 30 min, then sections were incubated with anti-STX6 antibody (1:1,000; Affinity Biosciences) overnight at 4°C. Then followed by incubation with HRP sheep anti-rabbit secondary antibody (1:5,000, Affinity Biosciences) for 30 min, sections were finally stained with hematoxylin. The degree of immunostaining was scored separately by two independent pathologists without any clinical pathologic data. The staining intensity scales were as follows: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellowish brown), and 3 (strong staining = brown). The scoring for the proportion of positive tumor cells were as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (10%~35% positive tumor cells), 3 (35%~70% positive tumor cells) and 4 (>70% positive tumor cells). The staining index (SI) was calculated by multiplying the staining intensity score and the proportional score of positive tumor cells. The SI range was determined as 0, 1, 2, 3, 4, 6, 9, 12 points to represent the expression level of STX6. According to this approach, SI ≥6 indicates a high expression level and SI ≤4 indicates a low expression level.

STX 6 Knockdown and overexpression

We used oligonucleotides(siRNAs) to knockdown *STX6* expression in SKOV3 cells, STX6 siRNA sequences #1:ATAGACAGGCACTGTGGGA, #2:CACACAGAGAGAAAATAA: STX6 plasmids, siRNA and control sequences were purchased from Guangzhou Ribo Biotechnology Company. SKOV3 cells were seeded in 6 well plates, and the medium was removed when cell confluence reaches ~80%, and freshly medium containing STX6 siRNA or STX6 plasmids and transfection reagent (Lipofectamine 2000, Invitrogen) was added into the plate. At 24 post-transfection, the medium was changed. Forty-eight hours later, cells were collected and the expression level of STX6 was detected by Western blotting.

Colony formation assay

SKOV3 cells were seeded in 6-well plates at 1,000/ per well and cultured in complete medium and PBS, siRNA #1, siRNA #2, control sequences were added into the plates, respectively. And the medium was changing every 24 hours. After 10 days of incubation under standard conditions, the extraction medium was removed and the cells were stained with 0.5% crystal violet solution in 20% methanol. After 10 min of staining, cells were fixed with paraformaldehyde after washing with PBS and photographed, and the number of cell clones was counted and analyzed.

Statistics

All data were analyzed using SPSS 22.0 statistical software, measurement data were expressed as mean ± standard error, one-way ANOVA was used to compare differences between groups, the relationship between *STX6* expression and clinic features was tested by Chi-square test (χ^2), P values < 0.05 were considered statistically significant. Each experiment was repeated at least three times.

Results

Relationship between STX6 expression *and prognosis*

The progression-free survival (PFS) data of 614 patients in Kaplan-Meier Plotter database was selected. According to the cut-off values, 276 patients were assigned to the STX6 high expression cohort, while 338 cases were assigned to the STX6 low expression cohort, and the total follow-up period was 250 months. The median survival in low expression cohort and high expression cohort was 19.33 months and 15 months, respectively. The results showed that the PFS in high *STX6* expression cohort was significantly reduced (HR=1.31; P=0.0051; Figure 1a). Taking overall survival (OS) as the observation index, 215 patients were assigned to the STX6 high expression cohort, while 440 patients were assigned to the STX6 low expression cohort, and the total follow-up period was 250 months. The median survival in low expression cohort and high expression cohort was 47.8 months and 38.93 months, respectively. Compared with low expression cohort, the OS in high *STX6* expression cohort was significantly reduced (HR=1.26, P=0.031; Figure 1b).

Relationship between STX6 expression and prognosis in 147 patients

To further reveal whether *STX6* expression correlates with patient prognosis, *STX6* expression levels in 147 ovarian cancer tumor tissues was detected by immunohistochemistry stain (Figure 2), and the correlation of *STX6* expression with PFS in patients was analyzed. According to the *STX6* expression score in immunohistochemistry, the patients were divided into high STX6 cohort and low STX6 cohort. There were 89 cases of high expression of STX6 and 78 cases of low expression, and the PFS was analyzed by long-rank test. The results showed that the PFS of the high expression group of STX6 was shorter than the low expression group (P=0.0016, Figure 3). The median survival of PFS in low expression cohort and high expression cohort was 31 months and 19.5 months, respectively.

Table 1. Relationship between *STX6* Expression and Clinicopathologic Features of Ovarian Cancer Patients

Variable	Total cases	High STX6 (n, %)	Low STX6 (n, %)	χ^2	p-value
Age (y)				0.053	0.817
≥50	87	52 (59.8)	35 (40.2)		
<50	60	37 (61.7)	23 (38.3)		
Differentiation				15.497	0
High	64	30 (46.9)	34 (53.1)		
Medium	39	22 (56.4)	17 (43.6)		
Low	44	37 (84.1)	7 (15.9)		
PCI				10.469	0.015
0	28	12 (42.9)	16 (57.1)		
1/10/2023	18	7 (38.9)	11 (61.1)		
11/20/2023	76	43 (56.6)	33 (43.4)		
>20	35	27 (77.1)	8 (22.9)		
Stage				11.41	0.01
I	26	10 (38.5)	16 (61.5)		
II	21	10 (47.6)	11 (52.4)		
III	68	44 (64.7)	24 (35.3)		
IV	32	25 (78.1)	7 (21.9)		

Relationship between STX6 protein expression and clinicopathological features

Next, we evaluated the relationship between *STX6* expression levels in ovarian cancer samples and the clinicopathological features of patients. According to the analysis of immunohistochemical staining of ovarian cancer slice samples, the expression level of STX6 was not related to the age of the patient (P>0.05), but was significantly related to the degree of tumor differentiation and tumor stage (P<0.05), higher STX6 was observed in the ovarian cancer with low differentiation degree or higher tumor stage. Moreover, it is interesting that *STX6* expression also has a significant correlation with peritoneal carcinomatosis index (PCI), and STX6 overexpression was observed in 77.1% patient when the PCI > 20 (Table 1). These results suggest that STX6 may promote the progression of epithelial ovarian cancer.

Expression of STX6 in fresh samples

Based on the analysis of the Kaplan-Meier Plotter database and retrospective clinical data, we found that high expression of STX6 significantly reduced the OS and PFS of patients (p<0.05). In order to further analyze the expression of STX6 in fresh ovarian cancer tissues, we further verified the RT-qPCR (Figure 4a) and Western blot (Figure 4b, 4c) on 13 fresh samples of OC, ovarian cancer

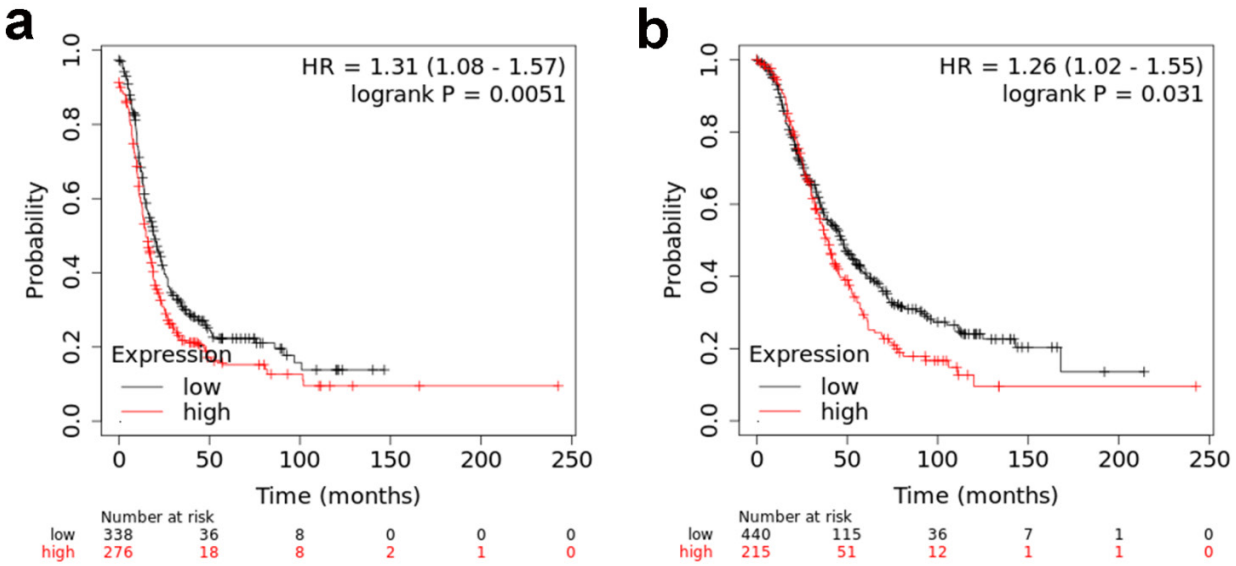

Figure 1. Kaplan-Meier Analysis of Ovarian Cancer based on Syntaxin 6 Expression. The ovarian cancer patient cohort was divided into quartiles based on STX6 expression. The cohort was then stratifed into 2 groups: High expression (top quartile) and Low/Medium (<top quartile); a. Kaplan-Meire curve was generated based on progression free survival (PFS) for ovarian cancer patients; b.Kaplan-Meire curve was generated based on overall survival (OS) for ovarian cancer patients

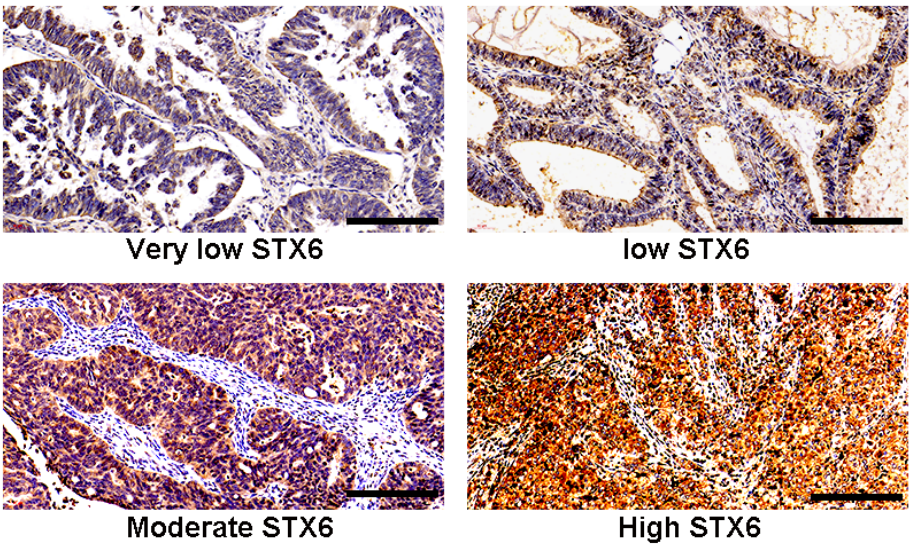

Figure 2. Representative Images of Different Expression Levels of *STX6* in Epithelial Ovarian Cancer Samples Detected by Immunohistochemistry

peritoneal metastases (OCPM) and 6 normal ovarian tissue samples. The results showed that the expression of STX6 in OC and OCPM was significantly higher than that in normal ovarian. Interestingly, the expression of STX6 in OCPM was significantly higher than that in the primary OC.

This result was consistent with the aforementioned phenomenon that *STX6* expression was positively correlated with PCI in patients.

Effect of STX6 on the proliferation of SKOV3 cells

In order to investigate the effect of *STX6* expression on the proliferation of ovarian cancer cells, we screened two different siRNAs and overexpressed plasmids to realize STX6 knockdown and overexpression in SKOV3 cell. Then western blot was used to verify *STX6* expression in different cells, while CCK8 and plate clone assay were used to detect the viability of SKOV3 cells. The results showed that plasmids could significantly promote *STX6* expression in cells, while siRNA#2 could significantly knock down *STX6* expression in SKOV3 cells (Figure 5a). The results of plate clones assay showed that the most tumor clones were observed in the STX6 overexpression group, and followed by the control group and the STX6 knockdown group, and the least number of clones was observed in the siRNA#2 knockdown group (Figure 5b). At the same time, the experimental results showed that the vitality was increased in STX6-overexpressed SKOV3 cells, while significantly decreased in STX6-knockdown SKOV3 cells (Figure 5c). These results show that STX6 significantly promote the proliferation of SKOV3 cells.

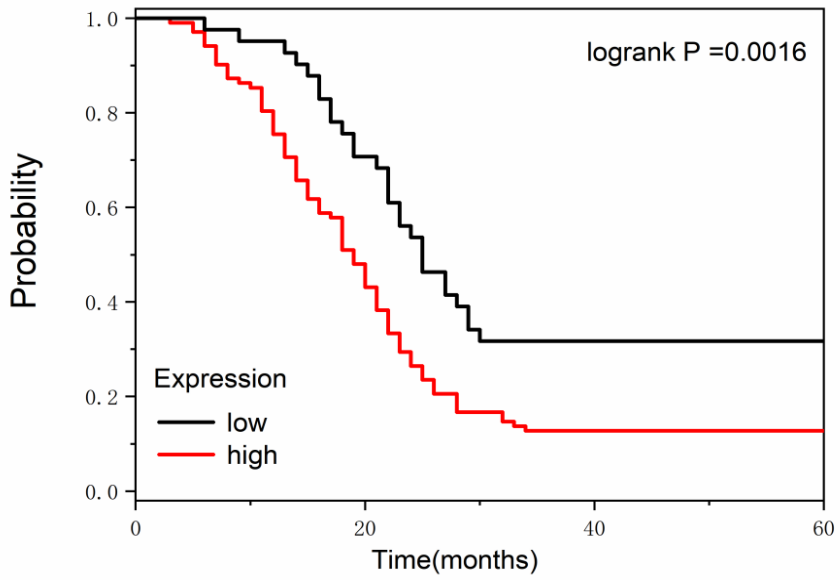

Figure 3 Kaplan-Meier Analysis based on PFS of Epithelial Ovarian Cancer Patients with Different *STX6* Expression Levels.

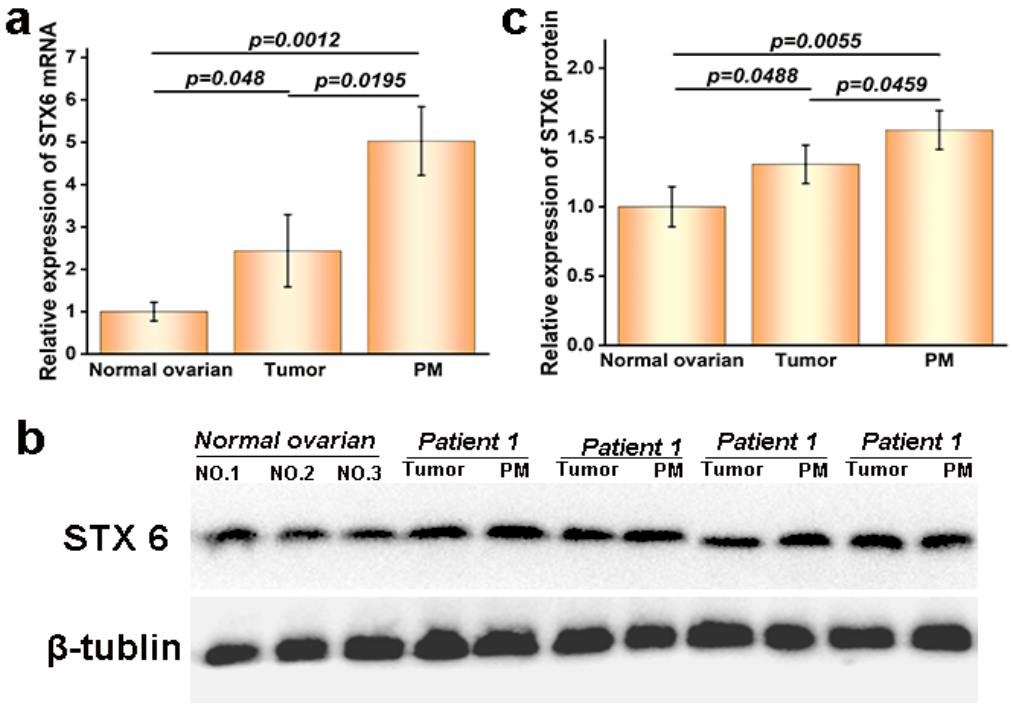

Figure 4 Expression Pattern of STX6 in Patient-Derived Normal Ovarian (NO), Ovarian Cancer OC, and Ovarian Cancer-Derived Peritoneal Metastases (OCPM). a. PCR detection of STX6 mRNA expression in NO, OC, and OCPM; b. Western blot detection of STX6 protein expression in NO, OC, and OCPM; c. Semi-quantitative analysis of STX6 protein expression in NO, OC, and OCPM.

Discussion

Epithelial ovarian cancer (EOC) is the sixth most common cancer in women and the most common cause of gynecologic cancer-associated death . Because the ovaries are located in the pelvis and the lack of typical symptoms of early ovarian cancer, it is difficult to diagnose ovarian cancer early, so that most ovarian cancer patients miss the

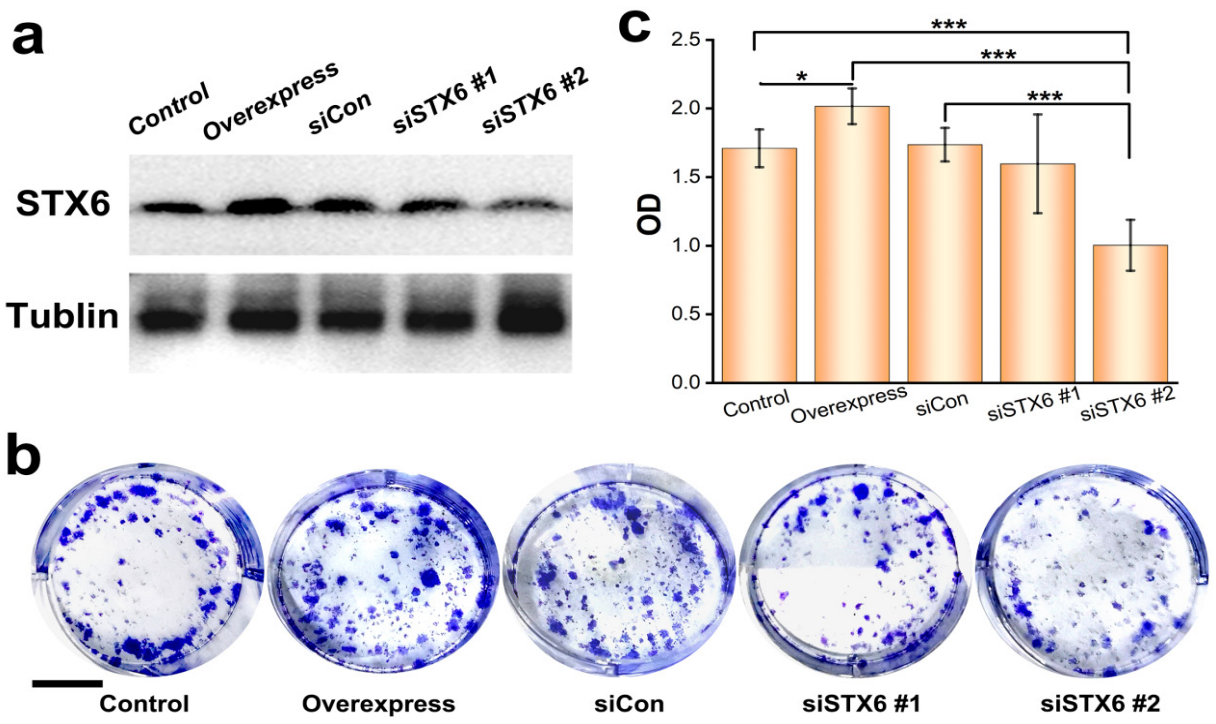

Figure 5. STX6 Promotes the Proliferation of Ovarian Cancer Cells. a. Western blot detection of STX6 protein expression levels in wild-type, STX6 overexpressed, and STX6-knocked SKOV3 cells; b.Clonal proliferation of wild-type, STX6-overexpressed, and STX6-knocked A cells; c.Cell viability of wild-type, STX6-overexpressed, and STX6-knocked A cells(*p<0.05,***p<0.001)

best treatment time. Ovarian cancer cells are easy to fall off and spread, resulting in rapid disease progression, so the 5-year survival rate of patients is still unsatisfactory (Yang et al., 2022). In order to improve the efficacy of patients, it is very important to explore the pathogenesis mechanism of ovarian cancer and explore the prognostic predictors of patients. In this study, Kaplan-Meier Plotter was used to analyze the prognostic relationship between STX6 and ovarian cancer, and the results showed that high expression of STX6 gene was associated with shortened OS and PFS in ovarian cancer patients. These results suggested that STX6 overexpression was a poor prognostic factor for ovarian cancer. These results were similar to the role of STX6 in renal cell carcinoma (Peak et al., 2019), prostate carcinoma (Peak et al., 2020), and esophageal carcinoma (Du et al., 2016) found in previous studies. In order to further verify the correlation between *STX6* expression and patient prognosis, the case data and postoperative specimens of 147 epithelial ovarian cancer patients who had undergone surgery were screened, and the expression level of STX6 in postoperative specimens was detected by immunohistochemistry. Retrospective analysis showed that there was no significant correlation between *STX6* expression level and age($P>0.05$). Instead, it was significantly correlated with the degree of tumor differentiation tumor stage, and PCI($P<0.05$), and STX6 was expressed higher in tumor tissues with lower differentiation, later stage and higher PCI. Although the relationship between tumor differentiation and stage has been confirmed in previous studies in tumors such as kidney cancer, this study further found that STX6 overexpression is associated with high PCI in ovarian cancer. The PCI evaluation system is widely used in peritoneal metastasis of colorectal cancer, but less commonly used in ovarian cancer (Quénet et al., 2021; van Baal et al., 2018). StålbergK's research showed that perioperative PCI was an excellent predictor of incomplete CRS and surgical complications for ovarian cancer and neoadjuvant chemotherapy should be considered when PCI > 24 (Stålberg and Jónsdóttir, 2020). Our study found a positive correlation between STX6 and PCI, suggesting that STX6 may be used for preoperative tumor burden assessment in ovarian cancer patients. In addition, we found that *STX6* expression levels in peritoneal metastases were higher than in the primary tumor in fresh samples. This additionally suggests that STX6 may be associated with the occurrence and progression of peritoneal metastasis in ovarian cancer. This may be that STX6 overexpression promotes vesicle transport and interaction between tumor cells, thereby promoting tumor cell proliferation. Further cell experiments have preliminarily verified that overexpression of STX6 can significantly promote the proliferation of tumor cells and the formation of clonal foci, while the proliferation of tumor cells is significantly inhibited after the use of siRNA knockdown of STX6. These results suggest that STX6 may play an important role in the occurrence and development of ovarian cancer and is expected to become a valuable diagnostic and therapeutic target. However, the shortcoming of this study is that the specific molecular mechanism of STX6 promoting tumor cell proliferation still needs to be further explored.

Author Contribution Statement

Wei Zhang collected data, analyzed data and wrote manuscripts, Qijun Lv designed and implemented experimental work, Donglin Lu participated in cell experimental work, Faqing Chen designed the research protocol and was responsible for this research.

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