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Clinical significance of SLP-2 in epithelial ovarian cancer and its regulatory effect on the Notch signaling pathway

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Abstract. – OBJECTIVE: To explore the expression of Stomatin-like protein 2 (SLP-2) and its clinical significance in epithelial ovarian cancer (EOC).

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the differential expression of SLP-2 in EOC tissues and cell lines. The relationship between SLP-2 expression and clinical pathological data of EOC patients was analyzed.

RESULTS: QRT-PCR results suggested that the SLP-2 was up-regulated in both EOC tissues and EOC cells by comparing with normal control. SLP-2 expression was a correlation with tumor pathological grade, distant metastasis, and TNM stage in EOC patients. Down-regulation of SLP-2 could significantly inhibit proliferation and promote apoptosis of EOC cells by activating the Notch signaling pathway. Knockdown of SLP-2 markedly downregulated Notch1 and Hes1.

CONCLUSIONS: SLP-2 was a novel factor involved in EOC progression, and could be utilized as a potential biomarker and therapeutic target for the EOC patients.

Key Words:
Epithelial ovarian cancer (EOC), Stomatin-like protein 2 (SLP-2), Notch signing pathway.

Introduction

Ovarian cancer (OC) is one of the common malignant tumors of the female reproductive system, and its morbidity rate is second only to that of cervical cancer and corpus carcinoma[1,2]. Epithelial ovarian cancer (EOC) is the most common subtype of OC. The mortality rate of EOC ranks first in all gynecological tumors, which seriously threatens women's lives[3]. After years of constant research and exploration, EOC has been confirmed to be a highly heterogeneous disease. Clinical manifestations of EOC are varied, posing great difficulties in treatment. Therefore, clarifying the mechanism of occurrence and development of EOC contributes to improve its prognosis.

SLP-2 is a member of the stomatin family[4]. Stomatin family members mainly include stomatin, SLP-1, SLP-2, SLP-3, and podocin[4]. SLP-2 is widely expressed in various human tissues[5-8]. Similar to stomatin, SLP-2 is also expressed on the surface of human erythrocyte membrane[5]. Furthermore, SLP-2 is highly expressed in human mitochondria[9], and it is able to regulate mitochondrial sodium-calcium exchange and participates in mitochondrial formation and function[10]. As a result, SLP-2 is able to improve the ATP storage and activates the ability of cells to resist apoptosis[11,12].

In the field of tumor research, the high-throughput screening results in multiple databases showed that SLP-2 is highly expressed in tumor tissues. In cDNA microarray screening results of colorectal cancer and normal paired tissues, it was found that the mRNA and protein expressions of SLP-2 are upregulated in tumor tissues[13]. In lung cancer cells, the expression of SLP-2 is upregulated compared to that of controls. Moreover, its level is associated with the age, tumor differentiation, lymph node metastasis, and clinical stage of lung cancer patients[14]. In endometrial cancer, the qRT-PCR results manifested that the mRNA expression of SLP-2 is also upregulated. Silence of SLP-2 in endometrial cancer cell lines could inhibit cell proliferation, while up-regulating its expression could promote tumor cell growth[15]. Sun et al[16] also showed that the elevated expression of SLP-2 is correlated with prognosis of EOC patients.

In this paper, we explored the potential biological function of SLP-2 in EOC and the underlying mechanism.

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Patients and Methods

Tissue Specimens

The tissue specimens were harvested from 70 EOC patients treated in our department from May 2016 to December 2018. In the meantime, 40 cases of normal ovarian tissues were harvested from uterine fibroid or adenomyosis patients of the same age as controls. During operation, the EOC tissues and normal tissues were taken and immediately placed into liquid nitrogen for RNA extraction. The general data of patients were shown in Table I. All patients had complete data and received no chemoradiotherapy before the operation. Moreover, this investigation was approved by the Ethics Committee of Women & Infants Hospital of Zhengzhou, and all patients signed the informed consent.

Cell Culture

The normal ovarian epithelial cell line (HOSE6.3) and the EOC cell line (OVCAR-3) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 100 μg/mL streptomycin, 100 U/mL penicillin (HyClone; South Logan, UT, USA) and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in the incubator with 5% CO_2 at 37°C under 95% saturated humidity. The cells in the logarithmic growth phase were used for experiments.

Transfection

Cells were inoculated into a 6-well plate (1×10^6 cells/well, 2 mL) and transfected with si-SLP-2 or si-NC (negative control) using Lipofectamine™ 2000 and OPTI-MEMI (Invitrogen, Carlsbad, CA, USA) at 80% confluence. The total RNA was extracted for later use after 48 h of transfection.

QRT-PCR Analysis

The total RNA was extracted from tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the AMV reverse transcription kit (2 μg total RNA added into 20 μL system). Later, Real-time PCR was conducted in a PCR instrument using the 2×SYBR Green PCR Mastermix (TaKaRa, Otsu, Shiga, Japan). Then, an appropriate amount of cDNA was taken as the template, and the corresponding forward and reverse primers were designed and synthesized according to the target gene, followed by amplification (primer concentration: 0.4 μmol/L, 15 μL system). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and three parallel samples were set for each sample. After three independent experiments, the data obtained were analyzed using $RQ=2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: SLP-2, F: 5'-GACAGCCAATAGCACTTAATAC-3', R: 5'-CCAAGGATGTCCGTAGGTT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Table I. SLP-2 expression and clinical features of patients with EOC.

Features	No.	SLP-2 expression		*p*
		High	Low	
No.	70	38	32	
Age (years)				0.870
< 50	24	15	9	
≥ 50	46	23	23	
Tumor size (cm)				0.793
< 10	31	15	16	
≥ 10	39	23	16	
Pathological grade				0.012
G1+G2	48	20	28	
G3	22	18	4	
Distant metastasis				0.009
No	50	21	29	
Yes	20	17	3	
TNM stage				0.014
I-II	33	10	23	
III- IV	37	28	9	

Western-Blot Assay

After transfection for 48 h, cells were lysed in an appropriate amount of lysis buffer to extract the protein, and the protein concentration was detected using the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). After loading on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a nitrocellulose membrane, and sealed with 5% skim milk powder overnight. Membranes were incubated with primary antibodies of SLP-2, Notch1, Hes1 and GAPDH at 4°C overnight. After the membrane was washed, membranes were incubated with corresponding secondary antibodies at 37°C for 2 h, followed by enhanced chemiluminescence (ECL) color development, exposure, and fixation. Finally, the gray value was detected.

Cell Apoptosis

Apoptosis was detected *via* Annexin V-FITC (fluorescein isothiocyanate)/Propidium Iodide (PI) double staining. After transfection for 48 h, cells were digested with trypsin and washed twice with pre-cooled phosphate-buffered saline (PBS), and the cell concentration was adjusted to 1×10^6 cells/mL. After the cells were suspended with binding buffer, the apoptosis in each group was detected according to the instructions.

Cell Proliferation

Cells were inoculated into a 96-well plate (2×10^4 cells/well, 100 μL) in an incubator with 5% CO_2 at 37°C for 24 h for synchronous treatment. After transfection, 10 μL of cell counting kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was added into each well for incubation for another 2 h. The absorbance (A) at 570 nm was measured using the microplate reader. The experiment was repeated for 3 times. Cell proliferation rate = $(A_{\text{transfection group}}/A_{\text{control group}}) \times 100\%$.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences between two groups were analyzed by the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Chi-square test was performed for the association between SLP-2 and clinicopathological parameters of patients. $p<0.05$ was considered as statistical significance.

Results

SLP-2 Was Up-Regulated in EOC

The expression of SLP-2 in tissues (70 cases of ovarian cancer tissues compared to 40 cases of normal ovarian tissues) was detected by qRT-PCR. The results showed that the average expression level of SLP-2 in 70 EOC tissues was (7.334 ± 0.571), which was significantly higher compared with 40 non-tumor tissues (1.649 ± 0.228) (Figure 1A).

The Relationship of SLP-2 Level with Clinicopathological Features of EOC Patients

Enrolled EOC patients were divided into SLP-2 high-expression group (n=38) and low-expression group (n=32) according to the mean expression level of SLP-2 (7.334). The association between SLP-2 expression and clinicopathological features of patients was analyzed. As shown in Table I, the expression of SLP-2 was not associ-

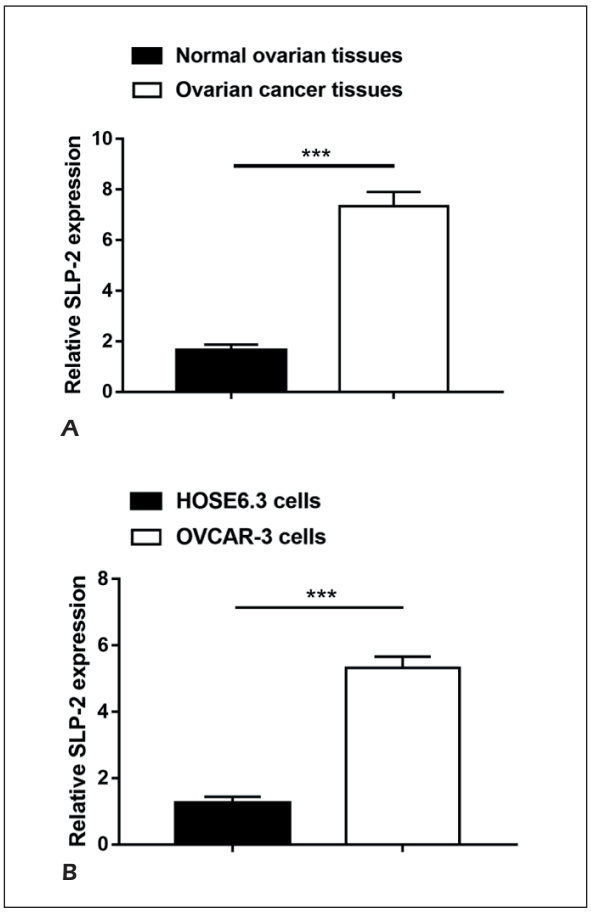

Figure 1. The expression of SLP-2 was measured in OC and normal tissues by qRT-PCR. **A**, In tissues. **B**, In cells. (***$p<0.001$).

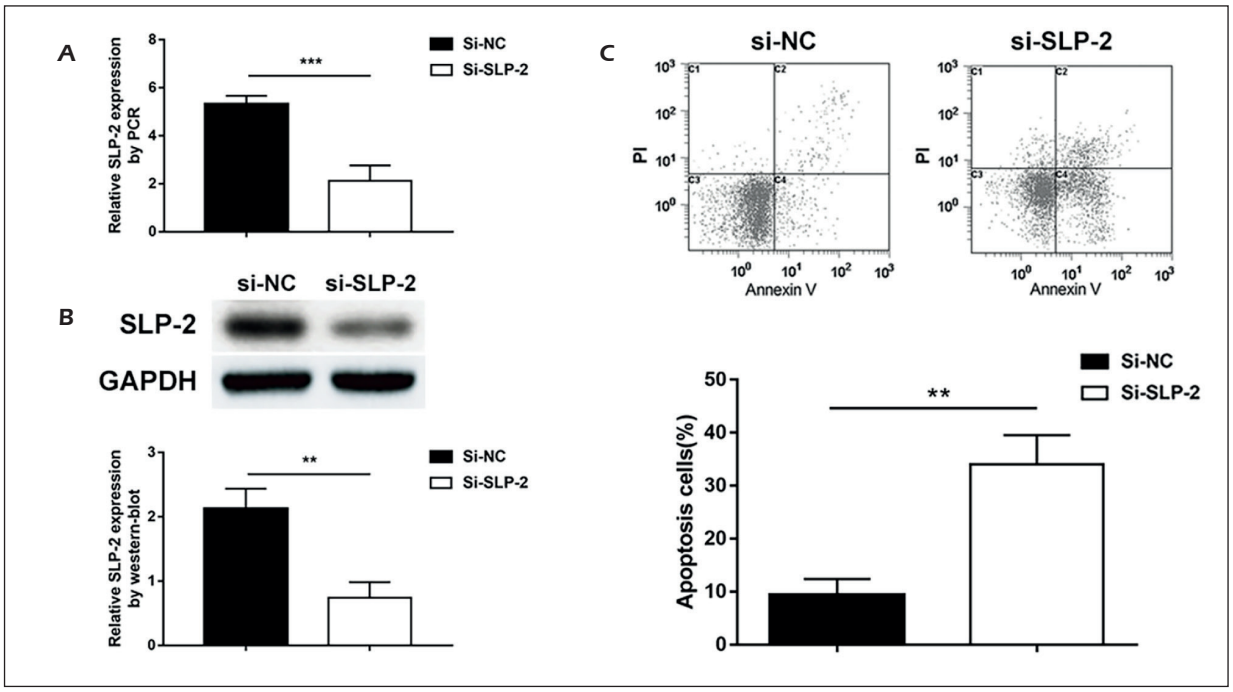

Figure 2. A-B, SLP-2 was efficiently knocked down by transfection of si-SLP-2. The expression of SLP-2 was detected by qRT-PCR and Western-blot. **C**, Cell apoptosis detected by flow cytometry. (**p<0.01, ***p<0.001).

ated with age and tumor size (p>0.05), However, significant statistical differences were found in tumor pathological grade, distant metastasis and TNM stage (p<0.05).

Downregulation of SLP-2 Expression Affected the Malignant Behavior of EOC Cells

First, we detected the expression of SLP-2 in OVCAR-3 cells and HOSE6.3 cells. The results were consistent with those in clinical samples (Figure 1B). The expression of SLP-2 in OVCAR-3 cells increased significantly, with an average expression of (5.404 ± 0.337), whereas the average expression of SLP-2 in HOSE6.3 cells was only (1.266 ± 0.254).

After OVCAR-3 cells were transfected with si-SLP-2 or si-NC, the expression of SLP-2 in cells was detected. The results from qRT-PCR and Western-blot showed that transfection of si-SLP-2 could significantly inhibit mRNA and protein expressions of SLP-2 in cells (Figure 2A-2B).

Flow cytometry was conducted to examine the regulatory effect of SLP-2 on cell apoptosis. As shown in Figure 2C, the apoptotic rate of cells with SLP-2 knockdown was significantly higher than that of normal cells.

CCK-8 assay was performed to detect the changes of cell proliferation. As shown in Figure 3A, compared with the control group, transfection of si-SLP-2 significantly inhibited the proliferation of ovarian cancer cells.

Downregulation of SLP-2 Expression Affected the Notch Signaling Pathway

Notch signaling pathway is activated in many malignant tumors. In our study, we obtained similar results that SLP-2 could activate the Notch signaling pathway (Figure 3). The two key proteins Notch1 and Hes1 in the Notch signaling pathway were down-regulated after knockdown of SLP-2 in EOC cells, indicating that SLP-2 was necessary for the activation of the Notch signaling pathway.

Discussion

Through *in vivo* and *in vitro* experiments, it was found that the expression of SLP-2 was significantly upregulated in EOC tissues. SLP-2 level was associated with tumor pathological grade, distant metastasis, and TNM stage of EOC patients, confirming the cancer promoting effect of SLP-2. SLP-2 is found to be upregulated in some

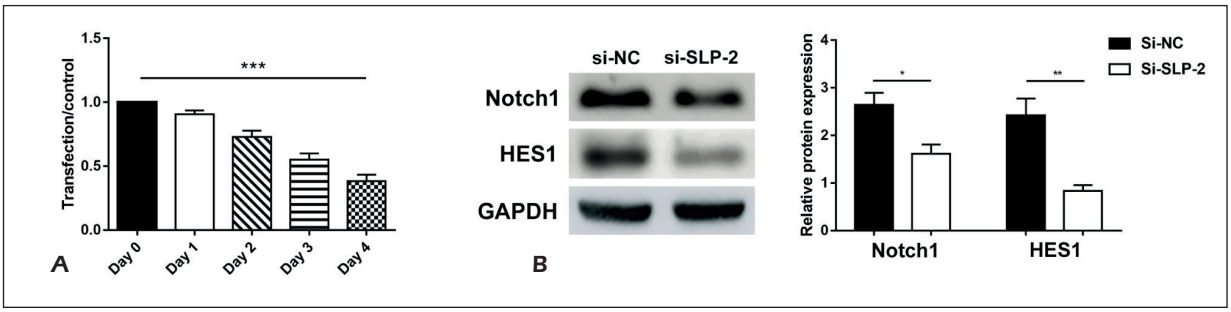

Figure 3. **A**, Cell proliferation ability detected by CCK-8 assay. **B**, The Notch signaling pathway was further analyzed, the expression of Notch1 and Hes1 was analyzed by Western-blot. (*p<0.05, **p<0.01, ***p<0.001).

other tumors as well[17-19]. The above results suggested that SLP-2 might play an important role in the occurrence and development of tumor.

In 2012, researchers pointed out that the elevated expression of SLP-2 in glioma indicates the poor prognosis. SLP-2 could promote the proliferation and metastasis of glioma cells by regulating the NF-κB/MMP9 signaling pathway[20]. In 2014, Liu et al[21] also found that SLP-2 is up-regulated in papillary thyroid carcinoma and can be induced by TGF-β. In our study, the effects of SLP-2 on the proliferation and apoptosis of EOC were further studied. Uncontrolled proliferation and abnormal anti-apoptotic ability are important reasons for the rapid expansion and spread of tumors[22]. Our findings uncovered that the proliferation ability of EOC cells was remarkably limited, while the number of apoptotic cells was significantly up-regulated after knockdown of SLP-2 in EOC cells.

The effect of SLP-2 on the Notch signaling pathway was determined, and it was found that silence of SLP-2 inactivated the Notch signaling pathway. The Notch signaling pathway is composed of Notch receptors and ligands, downstream effectors, and DNA-binding proteins. This pathway is highly conserved in evolution and can regulate cell proliferation, apoptosis, and body development from multiple aspects[23]. Notch1 is a receptor of Notch, and the Notch1 signaling pathway is abnormally activated in many tumors according to a large number of studies, such as lung cancer and glioma. Its abnormal expression can induce tumorigenesis directly or indirectly[24,25]. Hes1 is an important effector molecule in the downstream of the Notch1 signaling pathway as well as a marker for its activation[26,27]. In this paper, knockdown of SLP-2 in EOC cells down-regulated Notch1 and Hes1, suggesting the inactivated Notch signaling pathway.

Conclusions

To sum up, our research revealed that SLP-2 played an important role in the progression of EOC. High expression of SLP-2 was associated with advanced stage of OC. At the cellular level, down-regulation of SLP-2 could significantly limit cell proliferation and promote cell apoptosis of EOC by inactivating the Notch signaling pathway. However, the specific role and molecular mechanism of SLP-2 in EOC remained to be further studied.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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