Stomatin-like protein 2 is overexpressed and related to cell growth in human endometrial adenocarcinoma

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Abstract. Stomatin-like protein 2 (SLP-2) is a novel and unusual stomatin homologue of unknown functions. It was first identified to be overexpressed and involved in regulating cell growth and cell adhesion in human esophageal squamous cell carcinoma. We show herein the involvement of SLP-2 in human endometrial adenocarcinoma, and the effects of SLP-2 on endometrial adenocarcinoma cell growth. The expression of SLP-2 was evaluated in human endometrial adenocarcinoma by semi-quantitative RT-PCR, Westernblotting and immunohistochemistry. Sense and antisense SLP-2 eukaryotic expression plasmids were transfected into the human endometrial adenocarcinoma cell line HEC-1B. MTT assay and flow cytometry assay were performed to investigate the roles of the SLP-2 gene. SLP-2 was overexpressed in endometrial adenocarcinoma compared with their normal counterparts ($P \leq 0.05$). Immunohistochemistry showed that SLP-2 was mainly localized in the cytoplasm with some distribution on the membrane. HEC-1B cells transfected with antisense SLP-2 showed decreased cell growth, whereas the cell growth increased with the sense transfection. SLP-2 was first identified as a novel cancer-related gene overexpressed in human endometrial adenocarcinoma. Cell growth changes with the sense and antisense transfection revealed that SLP-2 might be important in endometrial tumorigenesis.

Introduction

Endometrial adenocarcinoma is the most common malignant disease of the female genital tract. It occurs during the reproductive and menopausal years, and recently its incidence is increasing. Multiple risk factors for endometrial cancer have been identified, such as endometrial hyperplasia, unopposed oestrogen exposure and adjuvant therapy for breast cancer using selective oestrogen-receptor modulators (SERMs), particularly tamoxifen. Oestrogen and SERMs are thought to be involved in endometrial carcinogenesis through their effects on transcriptional regulation (1,2). Obesity, nulliparity, and late menopause are also associated with endometrial cancer. During carcinogenesis of endometrial adenocarcinomas, multiple molecular alterations have been identified including micro-satellite instability (20-30%) (3), frequent mutations of PTEN (30-60%) (4-6), K-ras (10-30%), as well as ß-catenin (28-35%) (7). Traditional clinical pathologic prognostic factors include histologic type, grade, depth of myometrial invasion, angio-lymphatic invasion, lymph node involvement, and a few studies showed the relationship between specific molecular alterations and prognosis, such as p53 mutation, bcl-2 and cox-2 overexpression (8-11). However, the molecular pathways involved in the pathogenesis of endometrial adeno-carcinoma remain poorly defined.

SLP-2 is a novel and unusual member of the stomatin gene superfamily (12,13). The vertebrate homologues identified are SLP-1, SLP-2 and SLP-3 (12,13). SLP-1 is most abundant in brain and shares many similarities with UNC-24 (12,13). SLP-3 is specifically expressed in olfactory sensory neurons (12,15). All of these proteins as well as the stomatin from other species share a characteristic NH2-terminal hydrophobic domain as well as a consensus stomatin signature sequence that defines the stomatin gene family. Similar to other family members, SLP-2 shares the cognate stomatin signature sequence. However, it is the first member of this family to be recognized that lacks an NH2-terminal hydrophobic domain. These features distinguish it from stomatin, SLP-1 and SLP-3. SLP-2 may link stomatin or other integral membrane proteins to the peripheral cytoskeleton and play a role in regulating ion channel conductance or the organization of sphingolipid and cholesterol-rich lipid rafts (12). SLP-2 was first identified to be overexpressed in human esophageal cancer, transfection of antisense SLP-2 into esophageal cancer cells could reduce the cell growth and adhesion, which indicate that SLP-2 was a novel potential oncogene (14,15). To better understand SLP-2 expression status and the function of SLP-2 in human endometrial adenocarcinoma, we examined the expression of

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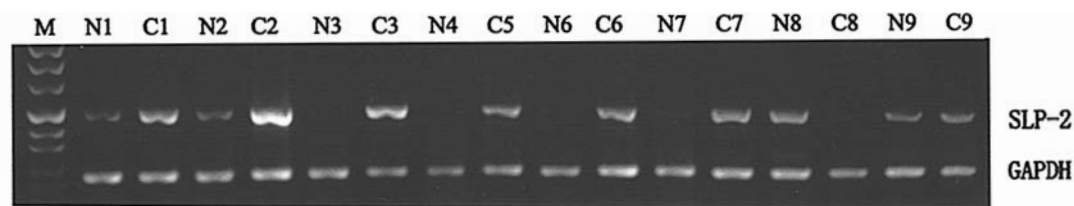*Key words:* endometrial adenocarcinoma; stomatin-like protein 2

Figure 1. Overexpression of SLP-2 in human endometrial cancer by semi-quantitative RT-PCR. SLP-2 is overexpressed in endometrial cancer (C) compared with their normal counterparts (N). GAPDH is used as an internal control.

SLP-2 in endometrial adenocarcinoma by semi-quantitative RT-PCR, Western blotting and immunohistochemistry. Sense and antisense-oriented SLP-2 was stably transfected into endometrial adenocarcinoma HEC-1B cells separately. The results showed that sense transfection of SLP-2 in HEC-1B accelerated cell growth whereas the antisense transfection reduced cell growth *in vitro*. The identification of SLP-2 as a novel cancer-related gene in human endometrial adenocarcinoma may shed light on endometrial tumorigenesis and may have some application for endometrial adenocarcinoma therapy in the future.

Materials and methods

Tissue specimens. Fresh tissue specimens were obtines from patients at the Department of Obstetrics and Gynecology, Affiliated Hospital of Medical College, Qingdao University (Qingdao, China) from April 2001 to August 2003 (Table I). The specimen were stored at -80˚C immediately after surgery until use. None of the patients had received radio- or chemotherapy before surgery. The subtype of endometrial carcinoma included: endometrioid 26 cases; adenosquamous 2 cases; clear cell 2 cases; squamous cell 1 case; and papillary serous 1 case; tumor limited to endometrium 8 cases. Tumor tissues were dissected from the resected specimens and the normal tissue was taken from the endometrium of patients with cervical cancer or CIN III. In addition, the sections used for immunohistochemistry were fixed in 4% polyformaldehyde and embedded in paraffin.

Cell culture. The endometrial adenocarcinoma cell line HEC-1B was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 μg/ml penicillin (pH 7.2-7.4) in a humidified incubator containing 5% CO_2 at 37˚C.

Semi-quantitative RT-PCR. Total RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of the RNA was assessed by 1% denaturing agarose gel electrophoresis and spectrophotometry. Total RNA (5 μg) of each sample was reverse-transcribed to the first strand of cDNA primed with oligo-(dT)12-18 using Transcriptase SuperScript II preamplification system for First Strand cDNA kit (Invitrogen). Then 0.5-1 μl aliquots of the cDNA was used as template to amplify SLP-2 fragment with primers: sense 5'-GTGACTCTCGACAATGTAAC-3'; antisense: 5'-T GATCTCATAACGGAGGCAG-3' at annealing temperature of 57˚C for 27 cycles. The relative expression of genes was determined using RT-PCR with GAPDH as an internal control.

Western blot analysis. Rabbit polyclonal SLP-2 antibody was previously made in our laboratory (15). For Western blot analysis, tissues were lysed with buffer (1% SDS, 10 mM Tris-Cl, pH 7.6, 20 μg/ml aprotenin, 20 μg/ml leupeptin and 1 mM AEBSF). The protein concentrations were determined using the BCA Protein assay kit (Pierce, Rockford, IL). Protein (10 μg) was separated on 12% of SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated with the appropriate anti-SLP-2 antibody (1:1,000 dilution) at 4˚C overnight. After washing, the membranes were incubated with secondary antibody at a dilution of 1:3,000 at room temperature for 1 h. Proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotechnology Inc.), and anti-ß-actin antibody (Sigma, St. Louis, MO) was used as loading control.

Immunohistochemical staining. Tissue sections were dewaxed with xylene and rehydrated through gradient ethanol into water. After endogenous peroxidase activity was quenched with 3% H_2O_2 for 30 min, sections were digested with 0.1% trypsin at 37˚C for 30 min. After PBS washes, non-specific antibody binding was blocked by preincubating slides in 10% normal goat non-immune serum at 37˚C for 30 min. Sections were incubated with the chicken polyclonal primary antibody against SLP-2 at 1:200 dilution for 2 h at 37˚C or overnight at 4˚C. Chicken SLP-2 antibody was previously made in our laboratory (15), sections were incubated with polymer helper at 37˚C for 30 min. After PBS washes, sections were incubated with secondary antibody at 1:200 dilution for 30 min at 37˚C, and developed using DAB (Sigma). Sections were washed in running tap water and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. Negative controls were obtained by omitting the primary antibody. SLP-2 expression was evaluated as described previously (15). The percentage of SLP-2 positive tumor cells was determined semi-quantitatively by assessing the entire tumor section. Each sample was assigned to one of the following categories: 0 (0-4%), 1 (5-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%). The intensity of immunostaining was determined as 0 (negative); 1+ (weak); 2+ (moderate) or 3+ (strong). A final immunoreactive score between 0 and 12 was calculated by multiplying the percentage of positive cells with the staining intensity score. All slides were evaluated for immunostaining without any knowledge of the clinical outcome of other clinical or pathological data.

Table I. Clinical features of the endometrial cancer cases.

Tissue diagnosis	Cases
Lymph node metastasis	
Absent	27
Present	10
Surgical staging	
I	23
II	4
III-IV	10
Differentiation	
Well	15
Moderate	16
Poor	6

Figure 2. Overexpression of SLP-2 in human endometrial cancer detected by Western blotting. ß-actin was used as a loading control. N, normal tissue; C, patient-matched tumor tissue.

SLP-2 transfection. SLP-2 sense and antisense plasmids in pcDNA3.1/myc-His(-) (Invitrogen) were constructed previously (15). The human endometrial adenocarcinoma cell line HEC-1B was transfected with sense, antisense construct using lipofectamine™ 2000 (Invitrogen), respectively. After G418 (400 μg/ml, Life Technologies, Rockville, MD) screening, the stable clones were identified by semi-quantitative RT-PCR.

MTT assay. HEC-1B cells transfected with sense and antisense SLP-2 expression vector were digested with trypsin and inoculated in 96-well plates at a concentration of $1x10^3$ cells/well after counting. After incubation at 37˚C in a humidified incubator containing 5% CO_2 for 1, 2, 3, 4, 5, 6, 7 days, MTT dissolved in RPMI-1640 at the final concentration of 0.5 mg/ml was added in a 96-well plate each day. The plates were incubated for an additional 4 h, then detected at 570 nm on Bio-Kinetics reader (Bio-Rad, Hercules, CA) after 200 μl of DMSO instead of RPMI-FBS was added to each well to solubilize the formazan crystals.

Flow cytometry assay. Flow cytometry assay was performed by propidium iodide staining. HEC-1B, sense and antisense SLP-2-transfected cells were grown to 80-90% confluence, then digested with trypsin, washed twice with PBS and fixed overnight at 4˚C in 70% ethanol. After being washed twice with PBS, cells were incubated with 5 μg/ml propidium iodide and 50 μg/ml RNase A in PBS for 1 h at room temperature. Flow activated cell sorter analysis was carried out using a FACSCalibur flow cytometer (Becton Dickson, Mountain View, CA) with CellQuest software. A total of 10,000 events were measured per sample.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software (SPSS Inc., Chicago, IL). The correlation between SLP-2 expression and clinicopathological characteristics or parameters was analyzed using Spearman's correlation analysis. Chi-square test was performed for comparison unless otherwise stated. P<0.05 was considered statistically significant.

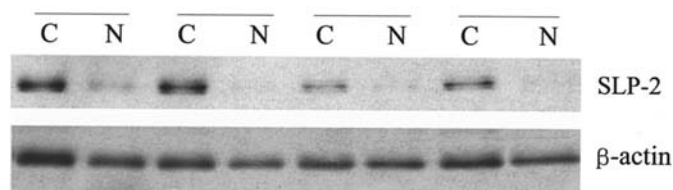## Results

Overexpression of SLP-2 in endometrial adenocarcinoma. RT-PCR analysis showed the differential expression of SLP-2 in endometrial adenocarcinoma tissue samples and the normal tissue samples. The gene expression of SLP-2 in endometrial adenocarcinoma tissue by RT-PCR was higher than that in the normal endometrial tissues (1.6±0.7 vs. 0.7±0.3, P<0.05, Fig. 1). There is no relationship between SLP-2 expression and the histological parameters at mRNA level. To confirm the overexpression of SLP-2 in cancer tissues at the protein level, immunoblot analysis of SLP-2 protein expression in patient-matched normal and tumor tissue from different individuals with endometrial adenocarcinoma was performed. SLP-2 was found to be up-regulated in 73% of endometrial adenocarcinoma tissues (27/37, Fig. 2).

Moreover, immunohistochemistry analysis was carried out to reveal the expression of SLP-2 in endometrial adenocarcinoma. SLP-2 was found up-regulated in 76% of endometrial adenocarcinoma (28 of 37, P<0.05) compared with their normal counterparts, respectively (Fig. 3). Strong positive staining was presented in cytoplasm of tumors (Fig. 3). However, no correlation was found between SLP-2 expression and the pathological characteristics.

Transfectants with antisense SLP-2 inhibits cell growth whereas transfectants with sense SLP-2 increases cell growth. After being transfected with antisense SLP-2, the expression of SLP-2 mRNA declined ~50%. MTT assay showed that cell growth of antisense stable clones was slower than that of HEC-1B or empty vector cells under normal culture conditions (Fig. 4). After transfection with sense construct, the expression of SLP-2 mRNA was increased by ~2.4-fold, and the cell growth was accelerated, compared to that of HEC-1B (Fig. 4). As inhibition of cell growth and proliferation can be the consequence of an induction either of apoptosis or of cell cycle arrest, flow cytometry assay was performed. No apoptosis was detected in sense and antisense SLP-2-transfected cells, while sense transfection resulted in an arrest at S phase by 8% and antisense transfection resulted in a G1 phase arrest by 10.8% as compared with parental cells or empty vector controls.

Discussion

Tumorigenesis is a complex and multistage process with many genes involved. To develop rational approaches to the diagnosis and treatment of cancer depends on identifying and understanding the molecular mechanisms that underlie
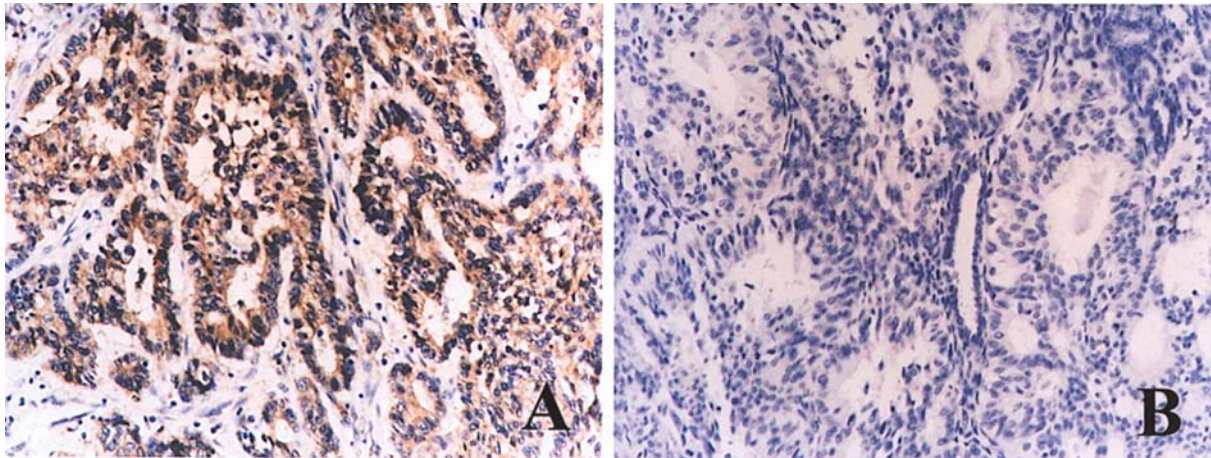
Figure 3. Immunohistochemical staining analysis of SLP-2 expression in human endometrial adenocarcinoma. (A) Strong positive staining of SLP-2 was found in plasma membrane and cytoplasm of human endometrial adenocarcinoma (x200); (B) negative control (x200). The sections were counterstained with hematoxylin.
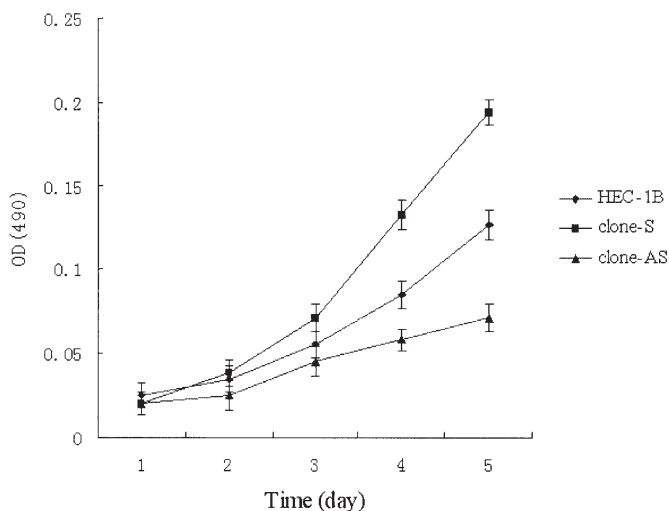

Figure 4. The effects of SLP-2 on HEC-1B cell growth. The cell growth decreased with antisense SLP-2 transfection, whereas the cell growth increased with the sense transfection. Clone-S, sense transfection clone; Clone-AS, antisense transfection clone; HEC-1B, parental cells.

tumor formation and progression. In this aspect, studies that seek to identify dysregulated genes and proteins in neoplasm are critical. Thus, research efforts aimed at systematically identifying the gene and protein expression profiles of normal and tumor cells are critically needed. The molecular events that lead to the development of endometrial cancer are poorly understood.

Human stomatin (band 7.2b) is a 31-kDa erythrocyte membrane protein of unknown function but implicated in the control of ion channel permeability, mechanoreception, and lipid domain organization. SLP-2 is encoded by a 1.5-kb mRNA. The gene for human SLP-2 is present on chromosome 9p13. Its derived amino acid sequence predicts a 38-kDa protein that is overall 20% similar to human stomatin. SLP-2 is also present in mature human erythrocytes. SLP-2 is a novel and unusual member of the stomatin gene superfamily that interacts with the peripheral erythrocyte cytoskeleton and presumably other integral membrane proteins but not directly with the membrane bilayer. SLP-2 may link stomatin or other integral membrane proteins to the peripheral cytoskeleton and thereby play a role in regulating ion channel conductances or the organization of sphingolipid and cholesterol-rich lipid rafts.

SLP-2 was first identified to be overexpressed in human esophageal cancer, reduced expression of SLP-2 in esophageal cancer cells could reduce the cell growth and cell adhesion, which indicate that SLP-2 was a novel potential oncogene. However, less is known about SLP-2 expression in other types of tumors including endometrial adenocarcinoma, herein we showed for the fist time that SLP-2 was also overexpressed in human endometrial adenocarcinoma at both mRNA and protein level. Furthermore, our results showed that sense transfection of SLP-2 in HEC-1B accelerated cell growth whereas the antisense transfectio reduced cell growth *in vitro*. The identification of SLP-2 as a novel cancer-related gene in human endometrial adenocarcinoma may shed light on endometrial tumorigenesis and may have some application for endometrial adenocarcinoma therapy in the future.

Acknowledgements

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