Promotion of the occurrence of endometrioid carcinoma by S100 calcium binding protein P

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Abstract

Background: Endometrial cancer, one of the most common malignant tumors, is a serious threat to women’s health. Endometrial hyperplasia is a precursor of endometrial cancer. S100 calcium binding protein P (S100P) has been found to play important roles in many types of cancer. The present study aimed to investigate the expression of S100P in endometrial cancer and its precursor lesions, and to explore the possible mechanisms.

Methods: We collected paraffin sections of normal endometrium, simple and complex non-atypical hyperplasia, atypical hyperplasia, and endometrioid carcinoma. The expression of S100P in endometrial cancer and its precancerous lesions was observed using immunohistochemistry. We also cultured primary endometrial cells and endometrial cancer cell lines (Ishikawa and RL95–2), and observed the expression of S100P in these cells. Laser confocal microscopy was used to observe the co-localization of S100P and its interacting protein Ezrin in RL95–2 cells. We employed lentiviruses to knockdown and overexpress S100P and then detected the F-actin distribution and cell invasion using phalloidin staining and Transwell assays.

Results: There was a gradual increase in the S100P signal as the disease progressed from normal endometrium and simple non-atypical hyperplasia, to complex non-atypical hyperplasia, atypical hyperplasia, and then to endometrial cancer. S100P was mainly distributed in the cytoplasm and co-localized with Ezrin in endometrial cancer cells. After knocking down S100P, F-actin aggregated in the nucleus or to the local cell membrane. Furthermore, knockdown of S100P in Ishikawa cells decreased their cell invasion capability. Meanwhile, S100P overexpression in endometrial stromal cells increased cell invasion.

Conclusions: These data suggested that S100P might be involved in the occurrence and development of endometrial cancer via interaction with Ezrin and re-organization of F-actin to promote cell invasion.

Keywords: S100 calcium binding protein P, Endometrial cancer, Ezrin, Cell invasion, Cytoskeletal rearrangement

Background

Globally, there are more than 380,000 new cases of endometrial cancer each year, which seriously threatens women’s health [1, 2]. In China, the incidence of endometrial cancer is also gradually increasing, with approximately 63,400 new cases diagnosed in 2015 [3]. Endometrial cancer (EC) has two recognized clinical subtypes: type I (estrogen-dependent, endometrioid carcinoma) and type II (non-estrogen-dependent, non-endometrioid carcinoma) [4]. Between them, type I accounts for more than 80% of the total number of cases [5].

Endometrial hyperplasia (EH) represents a series of morphological endometrial pathological changes that are at risk of developing into EC [6]. The WHO 1994...
classifications of EH categorized it as simple or complex hyperplasia, with or without atypia. The new WHO 2014 classification categorized EH as non-atypical EH and atypical EH [7]. Although EH with cytological atypia is characterized as a direct precancerous lesion of EC, simple hyperplasia shows a low risk of cancer progression, and complex hyperplasia has an intermediate risk of progression [6].

The incidence of EC is related to excessive exposure of the uterus to estrogens and the imbalance between estrogens and progesterone, which involves a series of molecules [5]. S100 calcium binding protein P (S100P) is a member of the S100 small molecular weight (9–14 kDa) calcium-binding protein family [8]. It is functionally implicated in many cancers, such as cholangiocarcinoma, breast cancer, and pancreatic ductal adenocarcinoma [9–11]. It participates in the regulation of proliferation, invasion, survival, metastasis, angiogenesis, and resistance to chemotherapy drugs to promote the occurrence and development of cancer [12–14].

In our previous studies, we found that S100P is expressed in normal endometrial cells and is upregulated in the endometrium at the mid-secretory phase, participating in the formation of endometrial receptivity [15, 16]. Recent studies suggested that S100P functionally improves the proliferation and invasion of EC [17, 18]. However, whether S100P is involved in the continuous progress from precursor lesions to type 1 EC, and its related mechanism, is unclear. In the present study, we investigated the expression of S100P in type 1 EC and EH, and demonstrated the effects of S100P on cell invasion and cytoskeletal remodeling.

Methods
Sample collection
Paraffin-embedded sections were collected from the tissue storage unit of the Obstetrics and Gynecology Hospital of Fudan University. The inclusion criteria included: Age 20 to 45 years old with EH or EC histologically confirmed by the Pathology Department of the Obstetrics and Gynecology Hospital of Fudan University. The exclusion criteria included patients that received hormonal therapy or chemotherapy within three months before sample collection. A total of 21 samples were collected. Among them, five samples were diagnosed as normal endometrium of proliferative phase, three were SH, five were CH, three were AH, and five were grade-1 type 1 EC.

Benign proliferative endometrium samples for cellular tests were obtained via curettage after hysteroscope or using a pipette catheter. The inclusion criteria included: Age between 20 to 40 years old, and with regular menstrual cycles (27–33 d). The exclusion criteria included: Patients with endometriosis, or with any other endometrial lesions, such as endometrial hyperplasia and polyps and intrauterine adhesions; patients with an intrauterine device or who received steroid hormone medication within 3 months.

RNA isolation and real-time PCR analysis
We used the Trizol reagent (Invitrogen, Waltham, Massachusetts, USA) to isolate total RNA and used RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) to generate cDNA according to the manufacturer’s protocol. Quantitative PCR reactions were performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) and detection was performed using the Applied Biosystem PRISM 7500HT system (Applied Biosystems, Foster City, CA, USA). In all samples, ACTB (encoding β-actin) was used as an internal reference to assess the expression of S100P. We use the online software Primer 3 to design primers at http://frodo.wi.mit.edu/, as shown in Table 1. All experiments were performed in triplicate.

Cellular immunofluorescence staining
Cell monolayers cultured in 96-well dishes or on coverslips were fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100 for 45 min, cells were incubated with 1:50 mouse anti-human S100P monoclonal antibody (R&D Systems, Minneapolis, MN, USA) as the primary antibody, and then incubated with 1:500 Cy3-labeled goat anti-mouse IgG (Invitrogen) as the secondary antibody. Alternatively, the cells were stained with 5 μg/ml FITC-phalloidin (Sigma) for F-actin staining. Cell nuclei were counterstained with Hoechst33258 stain (Sigma) at 1 mg/ml. The cells were then observed under a fluorescence microscope (Axiovert 200, Zeiss, Jena, Germany).
Table 1 Sequences of primers used in the present study

| Primer     | Forward                      | Reverse                      |
|------------|------------------------------|------------------------------|
| SI100P     | 5′- TACAGGCTTCTCGCAGTA-3′    | 5′- AGGGCATTTTGGTCTTG-3′     |
| ACTB       | 5′-CAGGACCTGACCTACCTA-3′    | 5′- TCAAAGAAGGTTACCGAATACT-3′ |
| SI100P-O    | 5′- CGGAATTTCTGATGACTACAGAC-3′ | 5′- CGGATCCGGCCATCT-3′       |
| SI100Pi-1   | 5′- CCGGAAAGGATGCCGCTGATAATTTATCCACGAGC-3′ |  |
| SI100Pi-2   | 5′- CCCTGCTGCTGATAATTGGTACTCACCTGAG-3′ |  |
| SI100Pi-3   | 5′- CCCTGCTGCTGATAATTGGTACTCACCTGAG-3′ |  |
| Scrambled  | 5′- CCGCTCTAGTTAAGTCGCCACCC-3′ |  |

* Primers for SI100P overexpression

b Sequences used for SI100P interference

e Sequences of the Scrambled control

Tissue Immunohistochemical staining

Paraffin-embedded sections were subjected to a two-step immunohistochemical approach. Formalin-fixed and paraffin-embedded sections (4 μm) were deparaffinized, hydrated, and heated in an oven for antigen retrieval, before being covered with 0.3% (w/v) hydrogen peroxidase for 30 min to inactivate the endogenous peroxidase. A rabbit monoclonal antibody was used as the negative control. Phosphate-buffered saline (PBS) instead of primary antibody. Antibodies were used as the primary antibodies. Phosphate-buffered saline (PBS) instead of primary antibody was used as the negative control.

Cell processing for laser scanning confocal microscopy

RL952 cells were seeded at 1 × 10⁵ per well into a 24-well plate in which coverslips had been preset into the wells. The cells were fixed in 4% paraformaldehyde after 24 h of culture to achieve an aggregation rate of about 50%. After permeabilization with 0.1% Triton X-100 for 45 min, rabbit anti-human Ezrin 1: 50 dilution (Epitomics, Burlingame, CA, USA; EP886Y) and mouse anti-human S100P 1: 50 dilution (R&D Systems) were used as the primary antibodies. DyLight 680 labeled Goat anti-rabbit IgG 1: 500 dilution (KPL, Gaithersburg, MD, USA) and DyLight 800-labeled Goat anti-mouse IgG 1: 500 dilution (KPL) were used as the secondary antibodies. Hoechst33258 solution (diluted with 5% fetal bovine serum PBS) at a concentration of 1:1000 was added for nuclei staining. The cells were viewed and scanned under a Leica Confocal scanning microscope (Wetzlar, Germany) after a fluorescent sealing tablet (Antifade mounting medium; Beyotime, Jiangsu, China) was added.

Lentiviral vectors construction and infection

The stuffer DNA was removed from psPAX2 (BD Pharmingen, San Diego, CA, USA) coated with Matrigel™ (BD Pharmingen) and selected in 2 μg/ml puromycin two days later. The efficiency of interference and overexpression was tested by immunofluorescence staining. Tissue Immunohistochemical staining, and the construct with the highest interfering efficiency of the three target sequences (SI100P interference-1) was used for the follow-up study. The raw data of SI100P overexpression and knocked-down plasmid sequencing were uploaded as supplementary data.

Cell invasion assay

Invasion of cells through 8-μm pores were assessed using a Transwell® Cell Culture chamber (Corning Costar, NY, USA) coated with Matrigel™ (BD Pharmingen) and selected in 2 μg/ml puromycin two days later. The efficiency of interference and overexpression was tested by immunofluorescence staining. The cells that moved through the pores were fixed in 4% paraformaldehyde and stained with 1% Giemsa blue (Sigma-Aldrich). The membranes were photographed and cells were counted in the central field of triplicate membranes.

Statistical analysis

Densitometric analysis was conducted to compare the expression level of proteins using Image J (Version 1.5.1; Image J, National Institutes of Health, Bethesda, MD, USA) and expressed as the ratio of target/ACTB.
NIH, Bethesda, MD, USA). The results were analyzed statistically using SPSS (Version 23, IBM Corp., Armonk, NY, USA). Results are presented as mean ± SD of three to six independent experiments. A T-test or one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons post hoc test, were performed to analyze statistical significance. * p < 0.05 indicated a statistically significant difference.

Results

S100P is highly expressed in endometrial cancer cells

The results of real-time PCR analysis showed that the S100P mRNA was highly expressed in RL95–2 and Ishikawa cells. Compared with primary endometrial epithelial cells, its expression was approximately 15-fold higher in Ishikawa cells and nearly 800-fold higher in RL95–2 cells (p ≤ 0.01) (Fig. 1B). The results of immunofluorescence showed that the S100P protein had a similar expression trend to the mRNA in endometrial cancer cells. The fluorescence signal intensities of S100P in these two cancer cell lines were significantly stronger than those of primary endometrial epithelial cells and stromal cells. Furthermore, the fluorescence signal in RL95–2 cells was much stronger than that in Ishikawa cells, and the difference was statistically significant. Fluorescent signals were distributed in the cytoplasm and nucleus, but mainly in the cytoplasm (Fig. 1A and C).

S100P gradually increases from complex, atypical hyperplasia, and to endometrial cancer

Immunohistochemical staining was performed to detect the expression and distribution of S100P in the tissue samples of endometrial cancer and its precursor lesions. The results showed that as the disease progressed, the S100P signals gradually increased. There was no significant difference between SH and the normal endometrial control; however, as the disease progressed to CH, AH, and EC, a significant increase in S100P levels was observed between the groups. The enhanced S100P signal was distributed in the stroma, glandular and

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**Fig. 1** Expression of S100P in endometrial cells. A: Immunofluorescence staining for S100P (red) and Hoechst33258 (blue) in EECs (a, b, c), ESCs (d, e, f), Ishikawa cells (g, h, i), and RL95–2 cells (j, k, l). PBS was used as a negative control (m, n, o), and the scale bar was 50 μm. B: The mRNA expression of S100P in different endometrial cells, as determined using qPCR. C: Fluorescence densitometric analysis using image J, normalized to that of EECs. EEC: primary endometrial epithelial cells, ESC: primary endometrial stromal cells, * P < 0.05
lumen epithelium, and accumulated mainly in the glandular epithelium (Fig. 2).

**S100P improves the invasion of endometrial cells**

The expression of S100P was inhibited by more than 95% at both the mRNA and protein levels after infection with the S100P shRNA lentiviruses, and the cells infected with scrambled sequence showed no significant change in S100P levels (Fig. 3A, B, C and D). We then overexpressed S100P in primary endometrial stromal cells. The results showed that the S100P level increased markedly in the transfected cells (Fig. 3E and F).

The results of cell invasion assays showed that the number of invading cells decreased significantly after S100P silencing in Ishikawa cells, while the number of invading cell increased after S100P overexpression in ESC cells (Fig. 4).

**S100P co-locates with EZRIN and causes F-actin rearrangement**

We used laser confocal microscopy to detect the colocalization of S100P and EZRIN in RL95–2 cells. The results suggested that the distribution of S100P and EZRIN in cells was consistent and co-localized (Fig. 5a). Phalloidin staining showed that F-actin fibers were evenly arranged in RL95–2 and Ishikawa cells. Knocking down S100P in both RL95–2 and Ishikawa cells caused F-actin fiber reorganization, in which they tended to aggregate to the nucleus or local membrane (Fig. 5B and C).

**Discussion**

In the present study, we revealed that S100P is involved in the progression of endometrial cells from precancerous lesions to endometrioid carcinoma. The significantly higher expression of S100P in endometrial cancer cells suggested that it might play important roles in endometrial cancer, as it does in other cancers [20, 21]. Furthermore, RL95–2 cells are derived from a grade 2 moderately differentiated adenosquamous carcinoma, while Ishikawa cells originate from a well differentiated human endometrial adenocarcinoma [22]. Therefore, the much higher expression of S100P in RL95–2 cells than in Ishikawa cells indicated that the expression of S100P might be related to the degree of malignancy of endometrial cancer. A recent article reported that the expression of S100P progressively increased from grade 1 to grade 3 endometrial cancers, which supported our hypothesis [17].

Moreover, the observation that S100P signals increased in CH and AH, indicated that S100P is expressed early in endometrial tumorigenesis and might play a positive role in the development and progression of endometrioid carcinoma. This contrasted with the opinion of Jiang, et al., who discovered that S100P was highly expressed in endometrial squamous cell and adenosquamous carcinomas, but not in adenocarcinoma [18]. These differences might be caused by the sensitivity of the experiments. In our study, the expression of S100P in adenosquamous carcinoma-derived RL95–2 cells was much higher.
than that in adenocarcinoma-derived Ishikawa cells, which supported Jiang’s opinion that S100P is overexpressed in endometrial adenosquamous carcinomas. However, the expression of S100P in adenocarcinoma-derived Ishikawa cells was also much higher than that in normal endometrial epithelial and stromal cells. Another study reported that the expression of S100P was elevated in endometrial cancer [17]. In that study, all the histological subtypes of samples examined were endometrioid, which supported our results. Regrettably, because of difficulties in sample collection, our sample size is small and we were unable to collect fresh samples from different endometrial hyperplasia for western blotting and real-time PCR verification. Further studies with expanded sample sizes are required to form more reliable conclusion.

Previous studies showed that S100P was localized in the cytoplasm and/or nucleus of a wide range of cells, and could also be secreted to extracellular regions [23–25]. Our results showed that highly expressed S100P mainly accumulated in the cytoplasm and was weakly detected in the nucleus in Ishikawa and RL95–2 cells. It has been reported that the intracellular component of S100P could interact with the cytoskeletal protein Ezrin, which plays pivotal roles in the rearrangement of the cytoskeleton and participates in cell movement [26, 27]. Our laser confocal microscopy results showed the co-
localized expression of S100P and EZRIN in endometrial cancer cells, which indicated that S100P might interact with Ezrin during the occurrence and progress of endometrial cancer. Interestingly, increased levels of Ezrin have also been reported in atypical endometrial hyperplasia and uterine endometrioid adenocarcinoma [28–30]. These reports further supported our hypothesis.

Ezrin is a principal member of the ERM (ezrin–radixin–moesin) protein family, which are known as
structural organizers that link membrane proteins to the underlying actin cytoskeleton [31]. Phalloidin staining demonstrated obvious reorganization of F-actin in both RL95–2 and Ishikawa cells after knockdown of S100P. This indicated that S100P might interact with Ezrin to influence the organization of F-actin in endometrial cancer cells. Rearrangement of F-actin fibers is crucial to the formation of membrane protrusions at the leading-edge during cell movement [32]. The results of the present study revealed that knockdown of S100P in both RL95–2 and Ishikawa cells caused F-actin to aggregate to the nucleus or local membrane, which might impede cell movement. The invasion assay confirmed that downregulation of S100P inhibited cell invasion, while overexpression of S100P enhanced it. Thus, S100P is likely to promote the invasion and progression of endometrial cancer.

**Uniqueness and limitation**

The uniqueness of this study is that we observed the S100P signal in EH and displayed the distribution of F-actin after S100P knockdown. However, the small sample size was our biggest limitation, and further expansion of the sample size is needed in future studies.

**Conclusions**

In summary, S100P is expressed highly in endometrial cancer cells, and increases gradually from normal endometrium and SH to CH, to AH and then to EC. S100P influences cell invasion by interacting with Ezrin and reconstructing F-actin, which implies its involvement in the occurrence and development of endometrial cancer.
Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-07350-x.

Additional file 1.

Abbreviations
S100P: S100 calcium binding protein P; EH: endometrial hyperplasia; EC: endometrial cancer; SH: simple non-atypical hyperplasia; CH: complex non-atypical hyperplasia; AH: atypical hyperplasia; EECs: endometrial epithelial cells; ESCs: endometrial stromal cells; DMEM: Dulbecco’s Modified Eagle Medium; BSA: bovine serum albumin; PBS: phosphate-buffered saline; ERK: ezrin–radixin–moesin

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Authors’ contributions
DZ and WZ contributed to the conception and design of the study. HZ and HK collected the samples. DZ, XC, and LW completed the experiments. BX and AZ contributed to the analysis of the data. All authors contributed to the interpretation of the data, and read and approved the final manuscript.

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Availability of data and materials
The raw data of S100P overexpression and knocked-down plasmid sequencing were uploaded as supplementary data. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (2015[23]), and written informed consent was obtained from all the participants in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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