Methylation status and protein expression of RASSF1A in endometriosis

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Abstract. Ras association domain family 1A (RASSF1A) gene inactivation by promoter hypermethylation is a common event in the development of a variety of types of human cancer. Accumulated evidence has demonstrated that DNA methylation serve a critical role in the pathogenesis of endometriosis. The aim of the present study was to analyze the methylation status and protein expression of RASSF1A in endometriosis (EMS). The ectopic and corresponding eutopic endometrium tissues were collected from 45 women with EMS (EMS group) and normal endometrium tissues from 20 women without EMS (control group). The methylation status of RASSF1A was examined by methylation specific polymerase chain reaction (MSP). Immunohistochemistry was performed to measure RASSF1A protein level in endometrium tissues. In normal endometrium samples, RASSF1A protein expression was significantly higher at the secretory phase than the proliferative phase. RASSF1A protein expression in the ectopic endometrium tissues and eutopic endometrium tissues were significantly reduced than in normal endometrium (P<0.05). The frequency of aberrant methylation of RASSF1A was 55.56% in ectopic endometrium and 33.33% in paired eutopic endometrium, whereas such methylation was not detected in normal endometrium. Moreover, RASSF1A promoter hypermethylation was frequently associated with reduced expression of RASSF1A, and was common in advanced stage in ectopic endometrium of EMS. Epigenetic inactivation of RASSF1A through aberrant promoter methylation may be important in the formation and progression of EMS, and assessment of RASSF1A methylation status in eutopic endometrium may be a potentially useful biomarker to enhance the early detection of EMS.

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Introduction

Endometriosis (EMS), a common, benign, estrogen-dependent disease affecting 5-10% of women of reproductive age, is characterized by the presence of ectopic endometrial glands and stroma outside of the uterine cavity, primarily in the peritoneum, ovaries and rectovaginal septum (1). Women suffering EMS may present with chronic pelvic pain, dysmenorrhea, dyspareunia and/or infertility. The incidence has been on the rise over the years (2). A number of theories, such as Sampson's theory (3), have been proposed to explain the etiology of EMS, but the exact mechanism of its development remains unknown.

The novel candidate tumor suppressor the Ras association domain family 1A (RASSF1A) gene has been recently identified and mapped to chromosome 3p21.3 (4). It was previously demonstrated that RASSF1A gene inactivation by promoter hypermethylation is a common event in the development of a variety of types of human cancer, for example in the lung, breast, ovary, endometrial and cervical carcinomas (5-7). The role of RASSF1A as a tumor suppressor gene has been suggested since exogenous expression of RASSF1A decreases *in vitro*-colony formation, suppresses anchorage-independent growth, and reduces the tumorigenicity of cancer cells *in vivo* (4,8).

EMS is a benign gynecological disease which displays malignant behaviors, such as enhanced proliferation and cell invasion, ectopic implantation of distant organs similar to tumor metastasis (9). Accumulating evidence has indicated that DNA methylation serves definite roles in the pathogenesis of EMS (10,11). It is therefore reasonable to hypothesize that epigenetic inactivation of RASSF1A may have a role in EMS. The aim of the present study was to investigate RASSF1A protein expression and the methylation status in the ectopic and eutopic endometrium of fertile women with EMS and normal endometrium of women without EMS by immunohistochemistry and methylation specific polymerase chain reaction (MSP) to better define the possible involvement of RASSF1A in the pathogenesis of EMS.

Materials and methods

Patients and samples. After informed consent was obtained, ectopic endometrium from the cyst walls of ovarian EMS

and the matched eutopic endometrium from the uterus were taken from 45 patients, aged 24-50 years. EMS stages were confirmed according to the Revised American Fertility Society Classification (12). For controls, normal endometrium samples were obtained from 20 healthy women, aged 34-48 years, who underwent tubal sterilization that was confirmed laparoscopically to be free of EMS. All of the patients underwent surgical treatment in the Department Gynecology and Obstetrics at Liaocheng People's hospital (Shangdong, China) between February 2012 and December 2013. None of these patients had received any gonadotropin-releasing hormone analogue, antibiotics, radio-, chemo-, or hormone therapy in the last 6 months prior to the surgery. All samples were histologically confirmed, and the phase of the menstrual cycle was determined by preoperative history and histologic examination. The mean ages of subjects were 41±2 years in the experimental group, and 38±2 years in the control group, and there were no significant differences between the two groups with respect to age. Each biopsy was divided in two fragments, which were either frozen in liquid nitrogen (and stored in a -80°C for DNA extraction) or fixed in 10% buffered formalin for histological confirmation of endometriosis and immunohistochemistry. The study was approved by the Ethics Committee of Liaocheng Hospital.

Immunohistochemistry. The expression of RASSF1A protein was evaluated by immunohistochemistry which was performed with a standard streptavidin-peroxidase (S-P) technique, using diamino-benzidine (DAB) as a chromogen. The procedures were as follows: tissue sections were dewaxed in xylene and gradient alcohol with decreasing concentrations. Antigen retrieval was performed using microwave irradiation at 750 W for 15 min in 10 mM citrate buffer (pH 6.0). After treatment with 3% H_2O_2 for 15 min, and 5% normal milk (Solarbio, China) for 30 min to block nonspecific protein binding. The slide were then incubated over night with anti-RASSF1A (mouse monoclonal IgG, clone 3F3, code number AB 23950, Santa Cruz Biotechnology, Dallas, TX, USA; 1:500 dilution). After 3 washes in PBS, sections were incubated with goat anti-mouse IgG-HRP (sc-2031, Santa Curz Biotechnology, Dallas, TX, USA; 1:1,000 dilution) in a streptavidin-peroxidase reagent (ThermoFisher Scientific, Inc., Waltham, MA, USA) for 15 min, then the peroxidase reaction was initiated by incubating tissue sections in DAB reagent (Beyotime Insititute of Biotechnology, Inc., Shanghai, China) for 10-20 min at room temperature. Sections were washed 3 times in distilled water. Slides were finally counterstained with Mayer hematoxylin (Beyotime Insititute of Technology, Inc.).

Brown staining was considered positive. Sections known to express RASSF1A were used as the positive control, and PBS was used as the negative control. RASSF1A protein expression appeared in the cytoplasm of the cells. Sections were observed under an optical microscope (Nikon, Tokyo, Japan) in x40 magnification. A total of 5 visual fields were randomly selected in each section, and they were graded at 4 levels according to the percentage of positively stained cells: negative (-), <5%; positive (+), 5-25%; positive (++), 25-50%; and positive (+++), >50%.

DNA extraction and bisulfite modification. DNA was extracted from frozen preserved tissues by proteinase K digestion (Beyotime Insititute of Biotechnology, Inc.) and a phenol/chloroform extraction method (Beyotime Insititute of Biotechnology, Inc.) (13), denatured with NaOH and treated with sodium bisulphate as previously described (14).

Methylation specific polymerase chain reaction (MSP) for RASSF1A. Promoter hypermethylation of the RASSF1A gene was determined by the MSP as previously described (13). PCR was performed using the primer sequences of RASSF1A listed below: The methylated reaction primer sequences were 5'-GTGTTAACGCGTTGCGTATC-3' (sense) and 5'-AACCCCGCGAACTAAAAACGA-3' (antisense), and the produce size was 93 bp. For the unmethylated reaction, the primer sequences were 5'-TTTGGTTGGAGTGTGTTAATGTG-3' (sense) and 5'-CAAACCCCACAAACTAAAAACAA-3' (antisense), and the produce size was 103 bp. Primers and PCR reagents were purchased from Shanghai Sangon Biological Engineering Technology (Shanghai, China). The PCR parameters was as follows: 94°C for 10 min; 40 amplification cycles of 30 sec at 94°C, 30 sec at the annealing temperature (59°C), 30 sec at 72°C, and finally 72°C for 10 min. Products were loaded onto 2% agarose gels (Santa Cruz Biotechnology, Inc.), stained with ethidium bromide (Beyotime Insititute of Biotechnology, Inc.) and visualized under ultraviolet illumination.

Statistical analysis. All statistical analysis were performed using the SPSS software, Version 13.0 (SPSS, Inc., Chicago, IL, USA). χ^2-test and Fisher's exact were used to compare RASSF1A protein expression and methylation status between the cases, and with menstrual cycle phase and clinical stage. Pearson's correlation analysis was used to evaluate the relationship between RASSF1A protein expression and methylaion status in EMS. P<0.05 was considered to indicate a statistically significant difference.

Results

Cycle phase. All surgeries were performed during the proliferative and secretory phase in 2 groups. In EMS group, surgery was performed during the proliferative phase in 21 patients and during the secretory phase in 24 patients. In the control group, surgery was performed during the proliferative phase in 5 patients and during the secretory phase in 15 patients. There were no significant differences between the 2 groups with cycle phase (χ^2=2.708, P=0.100).

The RASSF1A protein expression in different endometrium tissues. To determine RASSF1A protein expression in EMS, ectopic and eutopic endometrium samples from 45 patients with EMS and 20 controls without EMS were examined by immunohistochemistry. Immunohistochemical results showed that RASSF1A was mainly expressed in the cytoplasm of glandular epithelial cells in the endometrial tissues.

In normal endometrium samples, immunohistochemistry showed that RASSF1A protein expression was significantly higher at the secretory phase than at the proliferative phase (P<0.05). Either in ectopic or eutopic endometrium samples, the protein level of RASSF1A at the secretory phase was higher than at the proliferative phase, although this was not significant (P>0.05). The results are presented in Table I.

Table I. The protein expression of the RASSF1A in both ectopic and eutopic endometrium of EMS and normal endometrium of non-EMS and the relationship with the menstrual cycle phase.

Groups	n	RASSF1A protein expression				χ^2-value	P-value
		-	+	++	+++		
Ectopic endometrium of EMS	45	28	11	6	0		
Proliferative phase	21	15	4	2	0	1.420	0.233
Secretory phase	24	13	7	4	0		
Eutopic endometrium of EMS	45	18	17	9	1		
Proliferative phase	21	11	7	3	0	2.515	0.113
Secretory phase	24	7	10	6	1		
Normal Endometrium of non-EMS	20	3	4	7	6		
Proliferative phase	5	3	1	1	0		0.009
Secretory phase	15	0	3	6	6		

EMS, endometriosis; RASSF1A, Ras association domain family 1A.

Table II. The promoter hypermethylated status of the RASSF1A gene in ectopic and eutopic endometrium of EMS and the relationship with the menstrual cycle phase and clinical stages.

Groups	n	RASSF1A promoter methylation		χ^2-value	P-value
		M	U		
Ectopic endometrium of EMS	45	25	20		
Proliferative phase	21	14	7	1.969	0.161
Secretory phase	24	11	13		
Eutopic endometrium of EMS	45	15	30		
Proliferative phase	21	8	13	0.402	0.526
Secretory phase	24	7	17		
Clinical stages of EMS[a]	45	25	20		
Stage I, II	16	5	11	5.940	0.015
Stage III, IV	29	20	9		
Clinical stages of EMS[b]	45	15	30		
Stage I, II	16	11	5	1.751	0.186
Stage III, IV	29	14	15		

[a]in ectopic endometrium, [b]in eutopic endometrium. EMS, endometriosis; RASSF1A, Ras association domain family 1A.
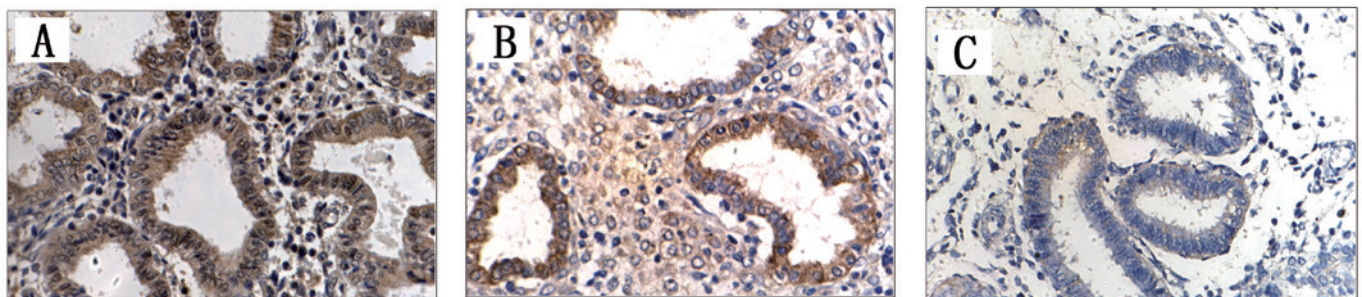

Figure 1. RASSF1A protein expression was assessed in different endometrium samples by immunohistochemistry. (A) A case of normal endometrium tissues from a woman without EMS demonstrating strong RASSF1A immunoreactivity. (B) A case of ectopic endometrium tissues from a women with EMS demonstrating moderate levels of RASSF1A immunoreactivity. (C) A case of eutopic endometrium tissues from a woman with EMS demonstrating weak RASSF1A immunoreactivity. EMS, endometriosis; RASSF1A, Ras association domain family 1A.

Table III. Correlation between the protein expression and methylation of RASSF1A in ectopic and eutopic endometrium of EMS.

		RASSF1A promoter methylation				
	RASSF1A	M	U	χ^2-value	r	P-value
Ectopic endometrium of EMS						
	+	3	14	15.901	-0.594	0.000
	-	22	6			
Eutopic endometrium of EMS						
	+	3	24	15	-0.577	0.000
	-	12	6			

EMS, endometriosis; RASSF1A, Ras association domain family 1A.
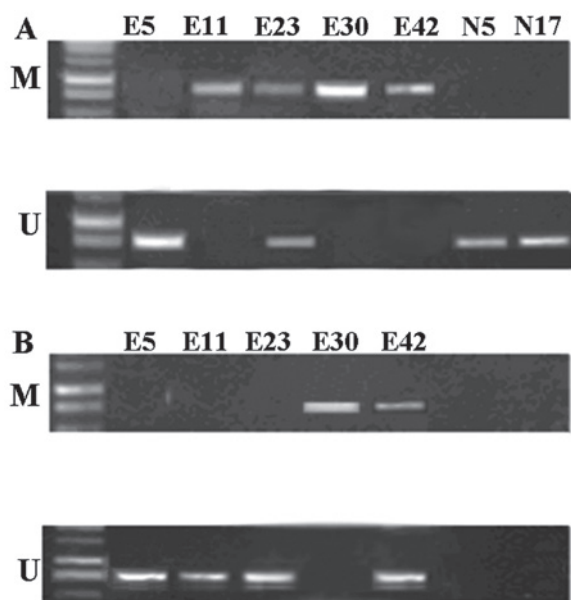

Figure 2. RASSF1A promoter hypermethylation status in EMS by MSP. (A) Promoter hypermethylation status of the RASSF1A gene in ectopic endometrium tissues.(B) Promoter hypermethylation status of the RASSF1A gene in eutopic endometrium tissues. M, MSP; U, unmethylated-specific PCR. E, endometriosis; N, normal endometrium samples. EMS, endometriosis; PCR, polymerase chain reaction; MSP, methylation specific PCR; RASSF1A, Ras association domain family 1A.

Fig. 1 demonstrated different degrees of RASSF1A protein expression in the 3 groups. The positive expression rates of RASSF1A in the endometrial tissues of each group were calculated and compared (Table I). The rates were 37.78% (17/45) and 60% (27/45) in the EMS ectopic and eutopic endometrium samples, respectively. In the control group, the positive expression rate was 85% (17/20). The expression level of RASSF1A was significantly lower in ectopic endometrium (χ^2=12.377, P<0.0001) and eutopic endometrium (χ^2=3.957, P=0.047) as compared with control subjects. The difference between ectopic and eutopic endometrium also was statistically significant (χ^2=4.447, P=0.035).

Methylation status of RASSF1A in different endometrial samples. To determine the correlation between DNA methylation and RASSF1A protein expression in endometrial tissues,

the methylation status of RASSF1A was evaluated in the above three endometrial tissues by using MSP. The results demonstrated that the promoter was hypermethylated in 55.56% (25/45) of RASSF1A genes in ectopic endometrium and 33.33% (15/45) in eutopic endometrium. In contrast, no RASSF1A promoter hypermethylation was observed in the control subjects. The difference was statistically significant between the above three groups (P<0.05). Thus, the RASSF1A promoter hypermethylation occurred exclusively in EMS. Representative examples are presented in Fig. 2.

As shown in Table II that frequency of promoter hypermethylation of RASSF1A did not vary significantly throughout the menstrual cycle either in ectopic endometrium or in eutopic endometrium.

The EMS stages were between stage I to IV. There were 16 cases of stage I and II, 29 cases of stage III and IV. As Table III demonstrates, in the ectopic endometrium, the frequency of promoter hypermethylation of RASSF1A at advanced stage (stage III, IV) was significantly higher than at early stage (stage I, II) (P<0.05). However, in the eutopic endometrium, frequency of promoter hypermethylation of RASSF1A at different clinical stages was not significantly different.

Correlation between RASSF1A protein expression and promoter hypermethylation. Finally, the correlation between RASSF1A immunoexpression and the methylation status in EMS was determined. In the ectopic endometrium, RASSF1A was methylated in 22 of 28 cases and negative for RASSF1A immunostaining. Meanwhile, in eutopic endometrium, RASSF1A protein expression was dramatically reduced in cases with RASSF1A promoter mythylation as compared with unmethylated cases. There was generally a negative correlation between hypermethylated RASSF1A and protein expression in both ectopic endometrium and eutopic endometrium. These data suggest that hypermethylation of RASSF1A promoter may be associated with abnormally low or absent expression in EMS. The results are shown in Table III.

Discussion

Epigenetics refers to heritable changes in DNA and chromatin that impact gene expression without changes in DNA sequence (15). There are 2 basic epigenetic regulatory

mechanisms: DNA methylation and histone modifications. DNA methylation is the best understood and most extensively studied epigenetic mechanism and refers to the covalent modification of post-replicative DNA, when a methyl group is added to the cytosine ring to form methyl cytosine (16). DNA methylation serves a critical role in the regulation of gene expression in development, differentiation, and complex diseases, cancer being the most prominent example (15,16). EMS shares some common features with cancer. For instance, the loss of the tumor suppressor PTEN in endometrial cancer was well-established in 2010 (17) and decreased PTEN expression has also been reported in the eutopic and ectopic endometrial of patients with EMS as well (18).

In women of reproductive age, EMS mainly involves the ovary (2); an endometriotic cyst of ovary is the most common type of EMS. Ovarian EMS was selected as the focus of the present study. Meanwhile, patients who underwent tubal sterilization that were confirmed to be laparoscopically to be free of EMS were selected as the control group.

The present study investigated the protein expression of RASSF1A in EMS. RASSF1A is localized in the cytoplasm of glandular epithelial cells in both ectopic and eutopic endometrium of EMS patients and in normal endometrium from women with non-EMS. It was demonstrated that RASSF1A was stronger expressed in normal endometrium. This is similar to Pallarés et al (19). In the present study, RASSF1A protein expression was significantly higher at the secretory phase than at the proliferative phase, but this is different from Pallarés et al (19), who found RASSF1A immunoexpression was significantly higher in the proliferative phase than in the secretory endometrium. This difference may be due in part to different patients and agents used.

In the present study, experimental evidence was presented that indicated that the frequency of methylation of the RASSF1A gene in ectopic endometrium and eutopic endometrium were 55.56 and 33.33%, respectively, whereas such methylation was not detected in normal endometrium. Combined with the immunohistochemical results, the positive expression rate of RASSF1A protein in the ectopic and eutopic endometrium were lower than in normal endometrium. In addition, a clear inverse relationship was observed between the extent of methylation in the RASSF1A promoter CpG island and its protein levels, either in ectopic or in eutopic endometrium. In addition, endometrium with RASSF1A methylation exhibited weak or lost RASSF1A protein expression in comparison to endometrium with RASSF1A unmethylation. Taken together, these results provide strong evidence that RASSF1A hypermethylation may constitute the mechanism through which RASSF1A expression is downregulated in endometriosis. Epigenetic inactivation of RASSF1A through aberrant promoter methylation may serve an important role in the pathogenesis of EMS.

The present data also demonstrated that there were statistical differences between eutopic and normal endometrium with regards to the methylation status and protein levels of RASSF1A. In addition, DNA methylation was observed in eutopic and ectopic endometrium of EMS. Eutopic endometrium is readily available and gene alteration in the eutopic endometrium can be easily detected. Identification EMS-related genes in eutopic endometrium will further reveal the pathogenesis of EMS and offer the basis for targeted gene diagnosis and therapy of EMS (20). Assessment of RASSF1A methylation status in eutopic endometrium of EMS may be a potentially useful biomarker to enhance the early detection of EMS.

It has previously been reported that hypermethylation of RASSF1A is associated with advanced tumor stage in bladder, esophageal, stomach and endometrial cancer (6,21-23). The severity of endometriosis has been clinically classified by numerous systems. The revised classification system of the American Society for Reproductive Medicine, currently widely used, consists of 4 stages (I-IV), with stage III and IV representing more serious cases (12). In the present study, comparing patients with early clinical stage to patients with advanced clinical stage there were statistical differences with regards to RASSF1A methylation in ectopic endometrium. However no statistically significant differences were observed in eutopic endometrium, which suggests that the methylation status of RASSF1A in the EMS eutopic endometrium tissues would not alter with clinical stage; promoter methylation of RASSF1A in the ectopic endometrium may therefore serve a more prominent role in the development and progression of EMS. This is a noteworthy topic that may merit further study.

Transient treatment of cells with demethylating agents can lead to a long-lasting demethylation effect (24). The epigenetic mechanisms represent a heritable dynamic and reversible means of modulating gene expression. The reversible nature of epigenetic aberrations is an important characteristic, since it allows us to search for appropriate pharmacological treatments, or 'epigenetic therapies' (25). In a number of studies it was demonstrated that RASSF1A expression was significantly increased or markedly upregulated in a number of types of cancer, including ovarian cancer and endometrial carcinoma, after treatment with 5-aza-DC (26-28). Therefore, DNA demethylation agents may be used in the future to restore methylation aberrations of RASSF1A in EMS.

However, how methylation of the RASSF1A promoter results in a reduction of protein expression in EMS needs further study. Further studies are necessary to confirm the findings in larger samples and to investigate the promoter in vivo prior to and following 5-aza-DC treatment.

In summary, the present study demonstrated, to the best of our knowledge, for the first time that the high frequency of promoter hypermethylation of the RASSF1A gene contributes to the loss or absent of protein expression in EMS. The results indicated that hypermethylation of the RASSF1A CpG island was involved in the pathogenesis of EMS. This also provides another piece of evidence that EMS may be ultimately an epigenetic disease. This may open up a novel avenue for the development of therapeutics for EMS and provide a potentially promising way for epigenetic reprogramming that restores dysregulated gene expression.

Acknowledgements

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