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Original Research

Relationship between the methylation levels of Twist gene and pathogenesis of endometriosis

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Abstract: The purpose of this study was to investigate the difference of Twist gene promoter methylation among ovarian ectopic endometrium, eutopic endometrium and non-endometriosis (EMs) endometrium . 15 patients with reproductive age hospitalized at Department of obstetrics and gynecology affiliated to Medicine School of Zhejiang University from January 2013 to June 2016 were selected. Among them, 5 patients underwent laparoscopic surgery due to ovarian type EMs, and were selected after histologic confirmation. Ectopic endometrium and eutopic endometrium were obtained simultaneously. Normal endometrium was obtained from 5 cases of tubal infertility confirmed by hysteroscopy. Six pairs of primers for CpG island of Twist gene promoter were designed, and the difference of promoter methylation levels was detected by pyrosequencing method for methylation specific PCR (MSP) in three groups of endometrial tissues. The promoter of Twist gene is hypomethylated in some areas of ovarian ectopic endometrium and eutopic endometrium of ovarian endometriosis. It is speculated that the regional hypomethylation of Twist gene promoter in ovarian ectopic endometrium and eutopic endometrium may cause over-expression of Twist protein, which may directly lead to the pathogenesis of endometriosis.

Key words: Twist; Ectopic endometrium; Eutopic endometrium; Non-endometriosis (EMs) endometrium.

Introduction

Endometriosis (EMs) refers to the presence of endometrial tissues (glands and stroma) with growth function in other areas outside uterine cavity coated endometrium and myometrium, which is one of the benign gynecological diseases (1, 2). It is a common disease in women of childbearing age, with symptoms of chronic pelvic pain, dysmenorrhea and even infertility, the incidence of which is as high as 10% to 15% (3). Endometriosis seriously affect women's health and quality of life and lead to 50% increase in risk of the epithelial ovarian cancer (2). However, the pathogenesis of the disease has not been fully clarified at present.

There is increasing evidence that epigenetic modification may be one of the important mechanisms in the pathogenesis of EMs (4). DNA methylation induces EMs by influencing hormone and micro-environment by changing the expression of regulating genes. DNA methylation refers to the covalent modification of a methyl group transferred to a specific base through a methyl donor SAM under the catalysis of DNA methyltransferase. In mammalian cells, DNA methylation is confined to the C-5 position of cytosine in dinucleotide of cytidylyl phosphate guanosine (CpG) and CpG dinucleotide aggregates to form CpG island. In the human genome, there is an 0.3Kb to 0.5Kb CpG island per 100Kb gene, which is generally located in the gene promoter region (5, 6). During human growth and disease development, methylation levels change and methylation degree vary from tissue to tissue (7, 8).

DNA methylation has been studied mostly in tumors and many canceration have also been found to be affected by oncogene methylation in gynecologic and obstetric tumors. Studies of P16 and CDH1 showed that there were differences in methylation expression between the tumor tissues of affinity breast cancer and the surrounding normal breast tissues. Moreover, the difference also exists between normal breast tissue around the tumor and normal breast tissue without tumor (9). De Leon et al. found that the hypomethylation of transmembrane protein 88 (TMEM88) promoter induced the high expression of TMEM88, which may be related to the mechanism of chemotherapeutic resistance (10). By qRT-PCR detection of mRNA and DNA methylation, Schmid et al. found that the gene promoter of the apoptosis inhibitor protein miR-34a was hypomethylated in different types of ovarian epithelial carcinoma, and its mRNA was highly expressed at transcriptional level (11).

The expression of COX-2 in patients with EMs was higher than that in eutopic endometrium and normal endometrium. The methylation of COX-2 gene promoter NF-IL6 in ectopic endometrium was lower than that in

normal and eutopic endometrium (12). It is inferred that the hypomethylation of NF-IL6 site of promoter may be related to the high expression of COX-2 in normal and eutopic endometrium. Andersson et al. used RT-PCR assay and methylation specific PCR (MSP) methods to study the expression of HOXA-10 promoter in 18 patients with EMs and 12 subjects with normal endometrium. It was found that the methylation of EMs eutopic endometrial promoter was significantly higher than that of EMs group and normal endometrium group (13). It is speculated that promoter methylation is involved in the pathogenesis of EMs.

Twist protein plays an important role in the regulation of cell differentiation and embryonic development. The previous study has shown that the expression of Twist increase significantly in ectopic endometrium (14). Abnormal expression of Twist protein contributes to the occurrence of EMs, and DNA methylation pattern can reflect the genes expression status. Twist has been confirmed to be a hypermethylated gene in breast and colorectal cancer, and Twist promoter has been shown to be hypomethylated (15) in gastric cancer, which leads to over-expression of Twist protein and mRNA. However, the role of Twist promoter methylation in the pathogenesis of EMs is unclear. Considering the possible role of promoter DNA methylation in the regulation of EMs expression, six pairs of primers were designed for PCR methylation analysis of the CpG island in the Twist promoter region to detect the difference of methylation status of Twist gene in ovarian endometrium, eutopic endometrium and non-EMs endometrium and to explore the root causes of differential expression of Twist proteins in different groups.

Materials and Methods

General data

15 patients with reproductive age hospitalized at Women's Hospital, Zhejiang University School of Medicine from January 2013 to June 2016 were selected. Among them, 5 patients underwent laparoscopic surgery due to ovarian type EMs, and were selected after histologic confirmation. Ectopic endometrium and eutopic endometrium were obtained simultaneously. Normal endometrium was obtained from 5 cases of tubal infertility confirmed by hysteroscopy. According to the r-AFS score, all the patients with ovarian type EMs were in III/IV stage, aged 25 to 40 years old, with median age of 33 years old. The median age of infertile patients was 31 years old, ranging from 26 to 39 years old. There was no significant difference in age between the two groups. Both groups were taken from proliferative endometrium and had no history of hormone therapy before 3 months. Tissue specimens were obtained under aseptic conditions and stored in -80 ℃ refrigerator.

Reagents and instruments

Genomic DNA extraction kit, methylation modification kit and PyroMark Q96 ID were purchased from QIAGEN company. The reagents of dNTP, TBE and loading buffer were purchased from Shanghai Geneland biotechnology Co., Ltd. Taq enzyme was purchased from KAPA company. Triton X-100 working fluid and DAPI were purchased from Sigma, USA and Abcam, UK, respectively. Anti-fluorescence attenuation sealant was purchased from Beijing Suoleibao. Sheep serum and Twist antibody were obtained from Beijing Zhongshan company and Abcam, USA, respectively. 4% paraformaldehyde, phosphate buffer solution (PBS) and bovine serum albumin (BSA) were purchased from Hangzhou Dawen, Shanghai Generay and Amresco, USA, respectively. Table model high speed centrifuge PICO17 and NANO-DROP were purchased from Thermo company. POWER PAC 3000 and DNA SUB CELL were purchased from BIO RAD company. Gel imaging system GIS-1600 and ABI 9700PCR system were purchased from Shanghai Tieneng Technology Co., Ltd. and Applied biosystems company, respectively. Laser confocal scanning microscopy (LCSM) was purchased from German Zeiss.

Endometrial stromal cell culture

After PBS rinsing, endometrial tissues were transferred into aseptic centrifuge tube and centrifuged at 1000r/min for 5 min at room temperature. The supernatant was discarded, 3ml collagenase was added to digest. After mixing by pipetting, the tube was put in a conical bottle, and digest for 1h at 37℃ in the 120r shaking bed. According to the condition of tissue digestion, DNase I enzyme was added timely to digest the tissue for about 20 min and mixed by pipetting for 5 min, so that the tissue mass could be blown away as much as possible. After 100 mesh and 40 mesh screen, the filtrate was centrifuged at 1000rpm for 8 min at room temperature. The supernatant was discarded and 2mL of the DMEM/F-12 medium containing 15% fetal bovine serum was added and mixed well, then inoculated to the six-well plate and cultured at 37 ℃ in a incubator with 5% CO_2.

Methylation modification

DNA was dissolved with sulfite reagent and 800μL water without RNA enzyme was added in each sulfite mixture, then mixed sufficiently. The following reagents (as shown in Table 1) were prepared in 200μL PCR thin-walled tubes with the total volume of DNA and water was 40μL. The mixture in the tubes were mixed thoroughly and placed at room temperature of 15°C to 25°C. The DNA transformation was performed by PCR instrument, and the reaction conditions were set according to Table 2 (about 5 hours). The PCR thin-walled tubes

Table 1. Composition of sulfite modification reaction.

Component	Volume (μL)
DNA solution (1-500ng)	Variable (up to 40μL)
RNase-free water	Variable
Sulfite mixture (dissolved)	85
DNA protective solution	15
Total volume	140

Table 2. Reaction conditions of DNA transformation by sulfite.

Step	Time	Temperature
Denaturation	5 min	95°C
Renaturation	25 min	60°C
Denaturation	5 min	95°C
Renaturation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Renaturation	175 min (2 h 55 min)	60°C
Hold	Indefinite*	20°C

were put in the PCR instrument and started the cycle.

Purification of sulfite modified DNA

After sulfite modification, 560μL of the freshly prepared Buffer BL (containing 10μg/mL carrier RNA) was added to each tube. The supernatant was removed after vortex mixing and briefly centrifugation. The EpiTect spin columns was placed in the collecting tube and the supernatant was removed to the column. Centrifuged for 1 min, the filtrate was discarded and the column was put back into the collection tube. 500μL of Buffer BW (wash buffer) was added to each column, then centrifuged at a maximum speed for 1 min. The filtrate was discarded and the column was put back into the collecting tube. 500μL of Buffer BD (demulfonic group solution) was added to each column and incubated at room temperature for 15 min, then centrifuged at the highest speed for 1 min, the filtrate was discarded and the column was put back into the collecting tube. Repeat the last step once. The column was placed in a 2mL collection tube and centrifuged for 1min at the highest speed to remove all residual liquids. The lid of the column was opened and placed into a new 1.5ml centrifuge tube, then incubated for 5 minutes at 56 °C. The column was placed in a new 1.5mL centrifuge tube, and 20μL of Buffer EB was added to the membrane center of the column, centrifuged at the maximum speed for 1min to elute purified DNA.

Methylation specific PCR

The primers were designed by PyroMark Assay Design 2.0 software and synthesized by Beijing Genomics Institute (Table 3). The MSP reaction system with total volume of 50μL was shown in Table 4. The number of PCR cycles was adjusted to 40 cycles so that the amplification was within the linear range. The PCR conditions were 'hot started' at 95°C for 3 min, followed by 40 cycles of 94°C for 30s, 51°C for 30s, 72°C for 1 min, and with a final extension cycle of 72°C for 7 min. The products of MSP were analyzed by agarose gel electrophoresis containing ethidium bromide (EB).

Pyrosequencing detection

The reaction bead (2μL), binding buffer (38μL) and PCR products (40μL) were added to the 96 well PCR reaction plate and mix well for 10 minutes at room temperature. The vacuum pump absorbs the binding beads and suspensions of PCR products, then immerses them into 70% ethanol, 0.2 M NaOH solution and flushing buffer successively for 5s. After the vacuum pump was turned off, the binding beads and PCR products on the

Table 3. Twist promoter methylation PCR primers.

Primer name	Primer sequence (5'to3')	5' modification
TWIST1-1F	GAAGTTGGAGGGTTGAGG	5'Biotin
TWIST1-1R	AACTAAACACCTCCTACATCATCTCT	
TWIST1-1S	ACACCTCCTACATCATCTCTC	
TWIST1-2F	GGGAGAGATGAGATATTATTTATTGTGTAG	
TWIST1-2R	AACAATTCCTCCTCCCAAACCATTCA	5'Biotin
TWIST1-2S	AGTTATTTAGGATGGGGT	
TWIST1-3F	GGGAGAGATGAGATATTATTTATTGTGTAG	
TWIST1-3R	CTAACAATTCCTCCTCCCAAACCATTC	5'Biotin
TWIST1-3S	GTTTAGGAGGGGAAGGAAA	
TWIST1-4F	GGGAGAGATGAGATATTATTTATTGTGTAG	
TWIST1-4R	TCTAACAATTCCTCCTCCCAAACCATTC	5'Biotin
TWIST1-4S	GGAGGGAGGTTAGGAG	
TWIST1-5F	GGGGAAAGGAGGGTTTAGA	
TWIST1-5R	ACCCACCCAATAATCAAATAAAC	5'Biotin
TWIST1-5S	AAGGAGGGTTTAGAAG	
TWIST1-6F	GTTTAGAAGGGAGAGAGAGTAGGT	5'Biotin
TWIST1-6R	ACCCACCCAATAATCAAATAAAC	
TWIST1-6S	ACCCCTTAAAATTCCAA	

Table 4. MSP reaction system.

Component	Dosage
H_2O	34.8 μL
5×buffer GC (KAPA)	10 μL
dNTP (10mM/each)	1 μL
Primer (forward 50pM/μL)	1 μL
Primer (reverse 50pM/μL)	1 μL
Template	2 μL
Taq (5U/μL)	0.2 μL

probe were placed in the 40μL annealing buffer (containing sequencing primers 1.5μL), denatured at 85℃ for 2 min. After cooling to room temperature, the primers were hybridized with template. According to the dose calculated by the sequence design information of Pyrosequencing software, the substrate mixture, enzyme mixtures and four dNTPs (QIAGEN) were added in the reagent tank in turn. The reagent tank and the 96-well reaction plate were put into the Pyrosequencing detector (PyroMark Q96 ID, QIAGEN) for the reaction. The methylation status of each site was analyzed automatically by the Pyro Q-CpG software.

Detection of cytoskeleton structure by immunofluorescence

After high temperature sterilization, the glass slide is evenly glued into the cell seed plate at the bottom of the culture plate. The cell density was about 60% by conventional culture, and the culture solution were changed after 8 h. After 72 h, it was rinsed with PBS buffer twice with each time of 2min. 4% paraformaldehyde was added at room temperature to fix cells, then rinsed with PBS buffer 3 times for each time of 2min. The specimens were treated with Triton X-100 working fluid for 5 min and then rinsed with PBS buffer 3 times for 2 min each time. The samples were treated with blocking solution for 30 min, then primary antibody was added and reacted at room temperature for 30min and incubated at 4℃ over night. Then the sample were rinsed three times with PBS buffer for 5 min each time. The second antibody was added for incubation avoiding light and reacted at room temperature for 60 min, then rinsed three times with PBS buffer for 5 min each time. The cells were dyed by DAPI for 5 mins avoiding light and washed twice for 2 min each time. Anti-fluorescence attenuating sealant was added to the slide and cover with the coverslip. Then the slide were observed under laser confocal scanning microscopy (LCSM) and analyzed.

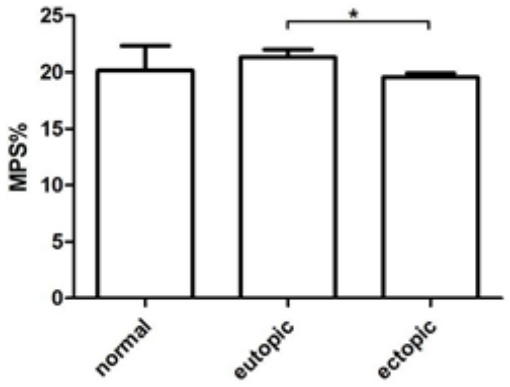

Figure 1. Twist MSP (pyrosequencing) analysis of Twist promoter methylation between ovarian type EMs, eutopic endometrium and non-EMs endometrium. The overall analysis of CpG5 loci showed that the methylation rate of EMs was the lowest, and the difference between EMs and eutopic endometrium was statistically significant ($P < 0.05$). There was no significant difference between the two groups of endometrium tissues and non-EMs endometrium ($P > 0.05$).

Statistical method

All the data of this study are statistical analyzed by SPSS 23.0 software. The measurement data were expressed as mean±standard deviation ($x \pm s$), and single factor analysis of variance (ANOVA) was used for comparison. LSD-t test was used for pairwise comparison. $P<0.05$ indicated that the difference was statistically significant.

Results

Twist promoter methylation difference between ovarian type EMs, eutopic endometrium and non-EMs endometrium was analyzed by MSP method

In this study, the difference of Twist promoter methylation between ovarian type EMs, eutopic endometrium and non-EMs endometrium was analyzed by MSP method. The primers of 6 CpG loci, namely Twist-1S, Twist-2S, Twist-3S, Twist-4S, Twist-5S and Twist-6S were designed for each group of 5 samples. Due to mismatch of the first locus (Twist-1S) in the methylation specific PCR (MSP) process, the remaining 5 loci were analyzed by pyrosequencing after MSP. The results showed that the mean methylation ratio of ovarian type EMs, eutopic endometrium and non-EMs endometrium was 19.55 ± 0.83, 21.33 ± 1.50 and 20.16 ± 4.90, respectively. There was significant difference in methylation ratio between ovarian type EMs and eutopic endometrium ($P < 0.05$), but there was no significant difference between them and normal endometrium ($P > 0.05$) (Fig. 1).

Low expression of Twist promoter methylation in EMs at Twist-5S and Twist-6S loci

The Twist-6S locus of promoter was analyzed and the results indicated that the methylation ratio of normal endometrium, eutopic endometrium and ovarian type EMs were 41.28±2.27, 44.61±1.071 and 38.37±3.134, respectively. LSD pairwise comparison revealed significant differences between the three groups ($P < 0.05$). At the Twist 5S locus of the promoter, the methylation ratio of normal endometrium, eutopic endometrium and

Table 5. Methylation ratio of CpG loci of Twist promoter in ovarian type EMs, eutopic endometrium and non-EMs endometrium.

CpG locus	Ovarian type EMs	Eutopic endometrium	Non-EMs endometrium	*P*
2S	26.52±5.35	29.40±5.30	33.28±9.53	P>0.05
3S	24.57±3.26	20.36±3.80	22.34±3.98	P>0.05
4S	19.39±4.24	20.55±4.38	18.00±7.04	P>0.05
5S	10.89±0.781	17.46±3.425	14.21±2.097	0.001
6S	38.37±3.134	44.61±1.071	41.28±2.27	0.004

ovarian type EMs were 14.21±2.097, 17.46±3.425 and 10.89±0.781, respectively (Table 5). There were significant differences between the three groups ($P < 0.01$). It can be seen that the methylation of Twist promoter in EMs is low in some CpG sites, which is significantly different from that of eutopic endometrium and normal endometrium. Collectively, these results suggested that the methylation of Twist promoter in EMs is low in some CpG loci, which was significantly different from that of eutopic endometrium and normal endometrium (Fig. 2).

Detection of Twist expression in endometrial stromal cells by immunofluorescence assay

In order to observe the Twist expression difference in endometrial stromal cells of ovarian type EMs, eutopic endometrium and non-EMs endometrium, the three kinds of cells were detected by Laser confocal scanning microscopy (LCSM) and immunofluorescence staining. The results showed that the expression of Twist in EMs and eutopic endometrial stromal cells was significantly higher than that in non-EMs endometrial stromal cells (Fig. 3). Because blue fluorescence represents the nucleus, green labeled Twist can only show cytoplasmic coloration, but nuclear staining is not clear. The stromal cells of EMs and eutopic endometrium were slightly larger than those of non-EMs endometrium. Morever, pseudopodia in stromal cells of EMs and eutopic endometrium increased compared with that in non-EMs endometrium (Fig. 3).

Discussion

Endometriosis (EMs) is one of the most common gynecological diseases that exist in endometrial glandular and stromal tissues. EMs has a high prevalence rate in women of reproductive age, which greatly influence the patients' quality of life. In recent years, with the development of molecular biology technology, the understanding of tumor and various diseases have changed. Because classical genetic changes such as mutation, deletion and translocation can not fully reveal the activation or inactivation of oncogenes, people have come to realize that epigenetics and other genetic mechanisms may be involved in the occurrence and development of cancer (16-18). The epigenetic modification of eukaryotes is mainly modified by DNA methylation, and its pathogenicity is attracting more and more attention. DNA methylation usually occurs at N-6 in adenine, N-7 in guanine, and C-5 in cytosine. In mammalian cells, DNA methylation is limited to the C-5 position of cytosine at cytidylyl phosphate guanosine (CpG) dinucleotide (19). DNA methylation plays an important role in inhibiting transcription, regulating chromosome structure, X chromosome inactivation, genomic imprinting, and so on. It is also involved in the formation of tumors and many diseases by affecting chromatin structure and the expression of oncogenes and tumor suppressor genes (20-23). Generally, about 50% of the CpG islands are located in the promoter region of the housekeeping gene and are unmethylated and active. When these CpG islands are methylated, the corresponding genes are silenced, while the other CpG islands in the genome do not affect the transcription of the genes (24, 25), whereas CpG sites outside the CpG islands are usually methylated. Previous studies have shown that the level of methylation often affects the degree of gene expression, which is usually negatively correlated, that is, when the methylation level is lower, the gene expres-

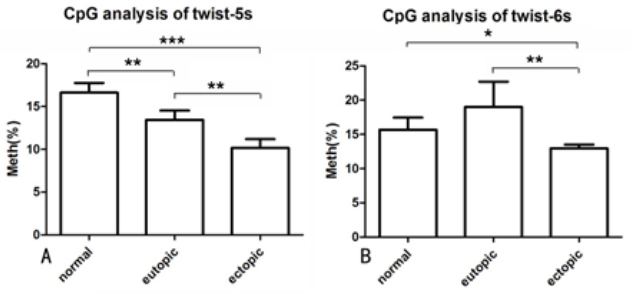

Figure 2. Twist promoter methylation in EMs at Twist-5S and Twist-6S loci. The methylation ratio at Twist-5S and Twist-6S loci of EMs was significantly decreased ($P < 0.01$). A. The methylation ratio of CpG 5S locus in the three groups was as follows: EMs < eutopic endometrium < non-EMs endometrium ($P < 0.01$). B. The methylation ratio of CpG 6S locus in EMs was the lowest, which was significantly different from that in eutopic endometrium and non-EMs endometrium ($P < 0.05$). * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.005$.

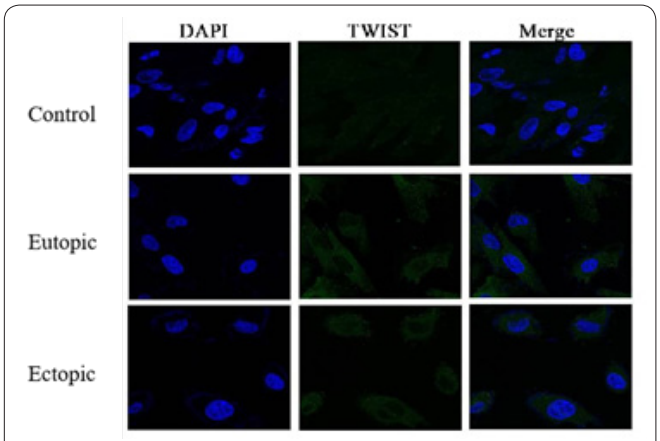

Figure 3. The expression and distribution of Twist in stromal cells of EMs, eutopic endometrium and normal endometrium were detected by immunofluorescence. Blue represents DAPI nucleus staining and green fluorescence represents Twist, which can only show cytoplasmic coloration. While merge of the two images shows colocalization in stromal cells.

sion level is stronger; conversely, when the methylation level is higher, the gene expression level is lower (26-28). The inactivation of tumor suppressor gene and mismatch repair gene caused by abnormal methylation, the gene activation caused by demethylation of proto-oncogene, and the instability of chromosome are all involved in the occurrence and development of tumor (29, 30).

Twist gene is located on chromosome 7p21.2, which acts as a transcription factor. The research on the function and mechanism of Twist protein is mainly focused on malignant tumor, which mainly shows potential carcinogenicity and belongs to oncogene. In a variety of tumors such as gastric cancer, bladder cancer, pancreatic cancer, and liver cancer (31-34), hypomethylation of gene promoter region resulted in increased transcription and protein expression, while hypermethylation decreased gene transcription and protein expression levels. In recent years, two pairs of primers have been used for methylation specific PCR (MSP) to detect specific methylation changes in a known gene (35). Two pairs of primers were designed for alleles specific to methylation and demethylation respectively. The chemically modified DNA sequences of methylated alleles could be distinguished from unmethylated alleles by PCR amplification. Because the DNA sequence of Twist gene was known, the primers were designed and amplified by MSP method and the methylation status of the target gene was detected. It has demonstrated that the expression of Twist is significantly increased in ectopic endometrium (14), while it is not clear whether the abnormal expression of Twist is associated with the pathogenesis of endometriosis.

This study is based on the fact that endometriosis (EMs) has the same problems of recurrence and metastasis as malignant tumors, so it is hoped that the detection of methylation status of Twist gene can provide a new method for the diagnosis and treatment of EMs. In this study, the methylation status of Twist gene promoter in ovarian type EMs, eutopic endometrium and non-EMs endometrium was detected by MSP method. It was found that some loci of ectopic and eutopic endometrium of EMs were hypomethylated, while those of normal endometrium were relatively hypermethylated. The results suggested that the methylation of Twist promoter may be the cause of high expression of Twist protein in EMs tissues and eutopic endometrium tissues, and then induce epithelial mesenchymal transformation, promote cell migration and invasion, and lead to the pathogenesis of EMs. There is regional hypomethylation in the promoter of Twist gene in EMs. It is inferred that this phenomenon is the direct cause of high expression of Twist mRNA and protein, thus inducing the pathogenesis of EMs, which is one of the important pathogenesis of EMs.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yang Jianhua; Fei Xiangwei, Zhang Tao, Ma Junyan, Zhou Jianhong, Lin Jun collected and analysed the data; Li Juanqing and Yu Hailan wrote the text and all authors have read and approved the text prior to publication.

Li Juanqing and Yu Hailan contributed equally to this work and should be considered as co-first authors.

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