Association of three single nucleotide polymorphisms of the E-cadherin gene with endometriosis in a Chinese population

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Abstract

Endometriosis, one of the most frequent diseases in gynecology, is a benign but invasive and metastatic disease. The altered expression of E-cadherin may play an important role in developing endometriosis. In this paper, we discuss the association of three single nucleotide polymorphisms (SNPs) on the E-cadherin gene and risk of endometriosis. We examined the genotype frequency of three polymorphisms in 152 endometriosis patients and 189 control women. There was a significant difference in the frequency of the E-cadherin 3'-UTR C→T genotypes between endometriosis and controls ($P=0.01$). The frequency of the C allele in patients (71.1%) was significantly higher than in the controls (63.8%; $P=0.04$). When compared with the T/T+T/C genotypes, the C/C genotype had a significantly increased susceptibility to endometriosis, with an adjusted odds ratio of 1.79 (95% confidence interval=1.17–2.76). No significant difference was found between endometriosis and control women on two polymorphisms (–160 C→A, –347 G→GA) at the gene promoter region of E-cadherin. The –160 C→A and –347 G→GA polymorphisms displayed linkage disequilibrium ($D'=0.999$). The –160 A/–347 GA haplotype was only detected in endometriosis patients (2%). These data show a relation between the E-cadherin 3'-UTR C→T polymorphism, the –160 A/–347 GA haplotype of two promoter polymorphisms and risk of endometriosis, suggesting a potential role in endometriosis development, at least in North Chinese women.

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Introduction

Endometriosis is one of the most frequent diseases in gynecology. The etiology and pathogenesis of this disease, defined as the ectopic location of endometrium-like glandular epithelium and stroma outside the uterine cavity, is unclear to date. Clinical observations have led to the assessment that endometriosis is benign gynecologically, but an invasive disease (Spuijbroek *et al.* 1992, Foidart *et al.* 1993). *In vitro* experiments also imply that endometriotic cells are invasive and able to metastasize. Analogous to tumor metastasis, it is likely that cell adhesion molecules are central for the invasion and metastasis of endometriotic cells.

Cadherins are a family of adhesion molecules that mediate Ca^{2+} -dependent cell–cell adhesion in all solid tissues of an organism and modulate a wide variety of processes, including cell polarization and migration (Takeichi 1987, 1990, Wheelock & Johnson 2003). E-cadherin, an epithelial cellular junction protein

expressed in almost all epithelial cells (Shimoyama *et al.* 1989), is among the best understood of the cadherins and plays an important role in the maintenance of epithelial development, organization, and cell integrity. The loss of E-cadherin, a metastasis suppressor molecule in carcinomas, is associated with tumor progression and metastases formation in a series of different cancers (Frixen *et al.* 1991, Birchmeier & Behrens 1994, Huiping *et al.* 2001, Yoshida *et al.* 2001). Is it possible that E-cadherin could also act as an invasion-suppressor molecule in endometriosis? Indeed, primary cells from human endometriotic biopsies but not from human endometrial biopsies were invasive in an *in vitro* collagen invasion assay. These *in vitro* invasive endometriotic cells were found to be nonmalignant epithelial cells lacking E-cadherin (Gaetje *et al.* 1997). Moreover, E-cadherin was slightly reduced in eutopic endometrium and significantly decreased in ectopic biopsies of endometriosis patients as compared with that in eutopic endometrium of unaffected women

(Starzinski-Powitz *et al.* 1999, Poncelet *et al.* 2002). Therefore, the loss of E-cadherin expression may constitute a crucial mechanism in the pathogenesis of endometriosis.

The E-cadherin gene is polymorphic; two adjacent SNPs ($-160\text{ C}\rightarrow\text{A}$ and $-347\text{ G}\rightarrow\text{GA}$) at upstream positions from the transcriptional start site of the promoter and a single base change from C to T at position $+54$ after the stop codon of the gene were identified (Becker *et al.* 1995, Li *et al.* 2002, Nakamura *et al.* 2002). These polymorphisms were associated with the development and progression of a certain cancer (Verhage *et al.* 2002, Shin *et al.* 2004a, Wu *et al.* 2005). The relation between E-cadherin gene polymorphisms and the risk of developing endometriosis has not been reported. In this paper, we studied three polymorphisms ($-160\text{ C}\rightarrow\text{A}$, $-347\text{ G}\rightarrow\text{GA}$, and $3'\text{-UTR } +54\text{ C}\rightarrow\text{T}$) in the E-cadherin gene in association with the risk of developing endometriosis, in North China.

Materials and Methods

Study participants

Blood was obtained from the following two groups and DNA was extracted for genotyping: (π) healthy female blood donors aged 25–51 years ($n=189$) and (θ) in patients for endometriosis in the Fourth Hospital, Hebei Medical University between 2001 and 2005 ($n=152$). The patients were all clinically, endoscopically, and histologically confirmed. All of the endometriosis patients who had not undertaken treatment with hormones were in stages III and IV. Patients were staged according to the revised American Fertility Society (1985) classification. General information on all patients was recorded in detail in the medical chart.

The control group consisted of women of reproductive age without any malignant disease, which was confirmed by surgical exploration at voluntary abortion, cesarean section, or pathologically after hysterectomy was performed for dysfunctional uterine bleeding. The general information on the healthy controls was extracted from their medical chart. All of the subjects were women of the Han nationality in North China. The Ethics Committee of Hebei Obstetrics and Gynecology Institute approved the study and informed consent was obtained from all recruited subjects.

DNA extraction

Venous blood (5 ml) was drawn from each subject into Vacutainer tubes containing EDTA and stored at 4°C . Genomic DNA was extracted within 1 week after sampling using proteinase K (Merck) digestion followed by a salting out procedure according to the method of Miller *et al.* (1988).

E-cadherin $-160\text{ C}\rightarrow\text{A}$, $-347\text{ G}\rightarrow\text{GA}$, and $3'\text{-UTR C}\rightarrow\text{T}$ genotyping

The E-cadherin $-160\text{ C}\rightarrow\text{A}$, $-347\text{ G}\rightarrow\text{GA}$, and $+54\text{ C}\rightarrow\text{T}$ genotypes were determined by PCR-restriction fragment length polymorphism assay. The primers for amplifying the E-cadherin promoter fragment were $5'\text{-CGCCCCGACTTGTCTCTCTAC-3'}$ (forward) and $5'\text{-GGCCACAGCCAATCA GCA-3'}$ (backward) and for the $+54\text{ C}\rightarrow\text{T}$ SNP at the E-cadherin $3'$ UTR were $5'\text{-CAGACAAAGACCAGGACTAT-3'}$ (forward) and $5'\text{-AAGGGAGCTGA AAAACCACCAGC-3'}$ (backward). The PCR was performed in a $25\text{ }\mu\text{l}$ volume containing 100 ng DNA template, $2.5\text{ }\mu\text{l}$ of $10\times$ PCR buffer, $1.5\text{ }\mu\text{l}$ of 25 mmol/l MgCl_2 , 2.5 U Taq DNA polymerase (BioDev-Tech., Beijing, China), $0.5\text{ }\mu\text{l}$ of 10 mmol/l dNTPs, and 200 nM each primer. The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 30 s at 94°C , 30 s at 66°C for C-160 A and G-347 GA, 30 s at 56°C for $+54\text{ C}\rightarrow\text{T}$, and 60 s at 72°C , with a final step at 72°C for 10 min to allow for the complete extension of all PCR fragments. The PCR product was 448 bp for the E-cadherin promoter and two $8\text{ }\mu\text{l}$ aliquots were subjected to digestion at 37°C overnight in a $10\text{ }\mu\text{l}$ reaction containing 10 U HincII (SBS Genetech Co. Ltd, Beijing, China) or 10 U BanII (TakaRa Biotechnology Co. Ltd, Dalian, China). After digestion, the products were separated on a 3% agarose gel that was stained with ethidium bromide. For the $-160\text{ C}\rightarrow\text{A}$ polymorphism, the A alleles were represented by DNA bands with sizes of 368 and 80 bp and the C alleles were represented by a DNA band with a size of 448 bp, whereas the heterozygotes displayed a combination of both alleles (448, 368, and 80 bp; Fig. 1). For the $-347\text{ G}\rightarrow\text{GA}$ polymorphism, the GA alleles were represented by DNA bands with sizes of 332 and 116 bp and the G alleles were represented by a DNA band with sizes of 263, 116, and 68 bp, whereas the heterozygotes displayed a combination of both alleles (332, 263, 116, and 68 bp; Fig. 2). The PCR product of the $3'\text{-UTR C}\rightarrow\text{T}$ polymorphism in the E-cadherin $3'$ UTR was 172 bp. After digestion by PmaCI (TakaRa Biotechnology Co. Ltd), the products were separated on a 3% agarose gel that was stained with ethidium bromide. As a result, the $3'\text{-UTR T}$ alleles were represented by a DNA band with a

Figure 1 E-cadherin $-160\text{ bp C}\rightarrow\text{A}$ genotyping by PCR-RFLP analysis followed by separation on 3% agarose gel as described in the text. Lane M = 100 bp ladder; lanes 2, 3, and 6 = C/C; lanes 1 and 5 = C/A; lanes 4 and 7 = A/A.

Figure 2 E-cadherin -347 bp $G \rightarrow GA$ genotyping by PCR-RFLP analysis followed by separation on 3% agarose gel as described in the text. Lane M=100 bp ladder; lanes 3 and 5=GA/GA; lanes 1 and 7=G/GA; lanes 2 and 6=G/G; lane 4=PCR product.

size of 172 bp and the 3'-UTR C alleles were represented by DNA bands with sizes of 146 and 26 bp, whereas the heterozygotes displayed a combination of both alleles (172, 146, and 26 bp; Fig. 3).

For a negative control, distilled water was used instead of DNA in the reaction system for each panel of PCR. The PCRs of 10% of the samples were run in duplicate for quality control.

Statistical analysis

Statistical analysis was performed using the SPSS10.0 software package (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg analysis was performed to compare the observed and expected genotype frequencies using the χ^2 test. A comparison of the E-cadherin -160 C $\rightarrow$ A, -347 G $\rightarrow$ GA, and 3'-UTR C $\rightarrow$ T genotype distributions in the study groups was performed by means of two-sided contingency tables using the χ^2 test. The E-cadherin -160 C $\rightarrow$ A and -347 G $\rightarrow$ GA haplotype frequencies and linkage disequilibrium coefficients were estimated using the EH linkage software (version 1.2, Rockefeller University, New York, NY, USA). The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model and adjusted by age accordingly. A probability level of 5% was considered significant.

Figure 3 E-cadherin 3'-UTR C $\rightarrow$ T genotyping by PCR-RFLP analysis followed by separation on 3% agarose gel as described in the text. Lane M=100 bp ladder; lane 2=T/T; lanes 3 and 4=C/T; lanes 5 and 6=C/C; lane 1=PCR product.

Results

Association of E-cadherin -160 C $\rightarrow$ A, -347 G $\rightarrow$ GA, and 3'-UTR C $\rightarrow$ T SNP with susceptibility to endometriosis

The distribution of the E-cadherin -160 C $\rightarrow$ A, -347 G $\rightarrow$ GA, and 3'-UTR C $\rightarrow$ T genotypes in the control group did not significantly deviate from that expected for a Hardy-Weinberg equilibrium ($\chi^2=0.33$, 0.14, and 2.65 respectively and $P=0.57$, 0.70, and 0.10 respectively). There was no significant difference in genotype and allelotype distribution of the E-cadherin -160 C $\rightarrow$ A SNP between endometriosis patients and controls (Table 1, all P values 0.05). When compared with the C/C genotype, the carriers of the 'A' allele were not significantly susceptible to developing endometriosis. The OR was 0.82 (95% CI=0.52–1.29; Table 2).

The frequencies of the three genotypes and two allelotypes of E-cadherin -347 G $\rightarrow$ GA in endometriosis patients were not different from those in controls (Table 1, all P values >0.05). When compared with the G/G genotype, the G/GA+GA/GA genotypes did not significantly influence the risk of developing endometriosis. The OR was 1.20 (95% CI=0.78–1.84; Table 2).

There were significant differences in genotype and allelotype distribution of the E-cadherin 3'-UTR C $\rightarrow$ T between endometriosis patients and controls (Table 1, all P values <0.05). The distribution of the C/C genotype in endometriosis patients (57.9%) was higher than that in the controls (43.4%). When compared with the T/T+C/T genotypes, the C/C genotype significantly increased the risk of development of endometriosis. The OR was 1.79 (95% CI=1.17–2.76; Table 2).

Table 1 Distribution of genotypes and alleles of three single nucleotide polymorphisms in E-cadherin genes.

Gene	Genotype/allele	Controls, no. (frequency)	Endometriosis, no. (frequency)	P (χ^2 test)
E-cadherin -160 C $\rightarrow$ A	Genotype C/C	121 (64.0)	104 (68.4)	0.61
	C/A	62 (32.8)	45 (29.6)	
	A/A	6 (3.2)	3 (2.0)	
	Allelotype C	304 (80.4)	253 (83.2)	0.35
	A	74 (19.6)	51 (16.8)	
E-cadherin -347 G $\rightarrow$ GA	Genotype G/G	108 (57.1)	80 (52.6)	0.26
	G/GA	71 (37.6)	57 (37.5)	
	GA/GA	10 (5.3)	15 (9.9)	
	Allelotype G	287 (75.9)	217 (71.4)	0.18
	GA	91 (24.1)	87 (28.6)	
E-cadherin 3'-UTR C $\rightarrow$ T	Genotype C/C	82 (43.4)	88 (57.9)	0.01
	C/T	77 (40.7)	40 (26.3)	
	T/T	30 (15.9)	24 (15.8)	
	Allelotype C	241 (63.8)	216 (71.1)	0.04
	T	137 (36.2)	88 (28.9)	

Table 2 Association of three single nucleotide polymorphisms in *E-cadherin* gene with the risk of endometriosis development.

Gene	Genotype	Controls (n)	Endometriosis (n)	OR (95% CI)
-160 C→A	C/C	121	104	0.82 (0.52–1.29) ^a
	C/A+A/A	68	48	
-347 G→GA	G/G	108	80	1.20 (0.78–1.84) ^b
	G/GA+GA/GA	81	72	
3'-UTR C→T	T/T+C/T	107	64	1.79 (1.17–2.76) ^c
	C/C	82	88	

^aThe odds ratio of the C/A+A/A genotype against the C/C genotype for -160 C→A polymorphism. ^bThe odds ratio of the G/GA+GA/GA genotype against the C/C genotype for -347 G→GA polymorphism. ^cThe odds ratio of the C/C genotype against the T/T+C/T genotype for 3'-UTR C→T polymorphism.

Haplotype of *E-cadherin* -160 C→A and -347 G→GA, two SNPs with susceptibility to endometriosis

The results of the 2LD program analysis showed that the *E-cadherin* -160 C→A and -347 G→GA polymorphisms displayed linkage disequilibrium ($D'=0.999$). The -160 C/-347 G was the most common haplotype in the control women (56.4%), followed by the -160 C/-347 GA (24.0%) and -160 A/-347G (19.6%) haplotypes. The -160 A/-347 GA haplotype was only detected in endometriosis patients (2%). When compared with the most common haplotype of -160 C/-347 G, the haplotypes of -160 C/-347 GA and -160 A/-347 G were not significantly indicative of the risk of endometriosis (Table 3).

Discussion

In the present study, we focused on the significance of *E-cadherin* polymorphisms to the risk of developing endometriosis. The results showed that there is no association between the -160 C→A and -347 G→GA polymorphisms at the *E-cadherin* gene promoter region and the risk of developing endometriosis. However, the haplotype of -160 C/-347 GA and the 3'-UTR C→T polymorphism of the *E-cadherin* gene

Table 3 Haplotypes of -160 C→A and -347 G→GA SNP in *E-cadherin* gene with the risk of endometriosis development.

Haplotype	Control n (%)	Endometriosis n (%)	OR (95% CI)
C/G	213 (56.4)	172 (56.6)	0.75 (0.49–1.15) ^a
A/G	74 (19.6)	45 (14.8)	
C/GA	91 (24.0)	81 (26.6)	1.10 (0.77–1.58) ^b
A/GA	0 (0)	6 (2.0)	

^aThe odds ratio of the A/G haplotype against the C/G haplotype in endometriosis. ^bThe odds ratio of the C/GA haplotype against the C/G haplotype in endometriosis.

were associated with the risk of endometriosis. The -160 C/-347 GA haplotype and 3'-UTR C/C homozygote indicated a relatively higher risk for developing endometriosis than other genotypes.

The -160 C→A and -347 G→GA are two common SNPs upstream from the transcriptional start site of the *E-cadherin* gene and have a significant effect on the transcriptional activity in transient transfection studies. Several major *cis*-acting elements have been identified within a short section of the proximal promoter. Among these are the two E boxes, a CAAT box and a SP1-binding site (Giroldi *et al.* 1997). The *E-cadherin* gene promoter thus exhibits a modular structure, suggesting that the strict control of epithelium-specific *E-cadherin* expression might result from interactions among the various regulatory elements (Behrens *et al.* 1991). Therefore, the molecular mechanism of differential transcriptional activity may be well explained as a difference in affinity of the DNA-binding protein(s) to the two allelic forms of the *E-cadherin* promoter. The 'A' allele of the -160 C→A polymorphism decreased the transcriptional efficiency by 68% when compared with the C allele (Li *et al.* 2002). The 'GA' allele of the -347 G→GA polymorphism decreased the transcriptional efficiency by tenfold when compared with the 'G' allele (Shin *et al.* 2004a). The -160 C→A and -347 G→GA allelic variation may be a potential genetic marker that can help identify individuals at a higher risk for invasive/metastatic diseases.

Recently, studies have investigated the association between the *E-cadherin* -160 C→A polymorphism and the risk of several types of cancer but the results were mixed. Verhage *et al.* (2002) found that 'A' allele carriers had a higher risk of prostate cancer when compared with 'C' allele carriers. Jonsson *et al.* (2004) studied Swedish patients with hereditary prostate cancer and also found a higher risk among 'A' allele carriers. However, there was no association between the *E-cadherin* -160 C→A polymorphism and the occurrence or progression of prostate cancer in Japanese populations (Tsukino *et al.* 2004). In a study of gastric cancer, Park *et al.* (2003) suggested that the -160 C→A polymorphism of *E-cadherin* has no direct effect on the risk of development of gastric cancer and its histological classification. However, the Wu *et al.* (2002) study suggested that individuals with the -160 A/A genotype of *E-cadherin* have a decreased risk of gastric carcinoma. The *E-cadherin* -347 G→GA polymorphism has been reported to be associated with risk of developing familial gastric cancer and sporadic colorectal cancers (Shin *et al.* 2004a, 2004b). The GA allele may be a risk factor for certain cancers.

In this paper, our study showed that there was no significant difference in the frequencies of the *E-cadherin* -160 C→A and -347 G→GA polymorphism genotypes between endometriosis and controls. However, the result shows a significant linkage disequilibrium between the -160 C→A and the -347 G→GA

polymorphisms, i.e. the -160 C allele tended to be linked to the -374 G allele. The -160 C/ -374 G was the most common haplotype in healthy women of North China (56.4%). The frequency of the -160 G/ -374 GA haplotype was 0% in the control group and 2% in endometriosis patients. The -160 G/ -374 GA haplotype may be a risk factor for developing endometriosis because the -160 G and -374 GA alleles were associated with decreased transcriptional efficiency of the E-cadherin gene. However, this result needs to be further tested in larger studies of endometriosis patients.

The C $\rightarrow$ T polymorphism is located at nucleotide 2797 of the E-cadherin cDNA sequence, 54 nucleotides downstream from the TAG stop codon. Although the function of the 3'-UTR +54 C $\rightarrow$ T polymorphism was unclear, studies have showed that this polymorphism was associated with cancer and other diseases (Tsai *et al.* 2003, Wu *et al.* 2005, Lin *et al.* 2006). Wu *et al.* found that the 'CC' homozygote indicates a relatively higher risk for developing prostate cancer than other genotypes (Lin *et al.* 2006). Our study also showed that the frequency of the 'CC' homozygote in endometriosis patients (57.9%) was higher than that in controls (43.4%) and the 'CC' homozygote carriers had a higher incidence of developing endometriosis (1.79-fold) than those who do not. Our other study was consistent with this paper, which suggested that the 'CC' homozygote was a high risk factor for the development of gastric cardiac adenocarcinoma and ovarian cancer. In addition, the E-cadherin protein expression of ovarian cancer patients with CC genotype was significantly lower than that of the ones with CT or TT genotype ($P=0.017$; unpublished results). As we know, the 3'-UTR is not the translated area of the protein, but it may be associated with mRNA stability of the transcript. The understanding of the E-cadherin gene 3'-UTR is limited. The Keirsebilck *et al.* (1998) *in vitro* study revealed that the downregulation of E-cadherin protein expression was caused by mRNA instability that was triggered by particular 3'-UTR sequences. Moreover, there are many examples where the 3'-UTR may change the expression of genes, such as the *TYMS* gene that contains a 6 bp deletion/insertion in the 3'-UTR that affects the *TYMS* mRNA stability and translation (Ulrich *et al.* 2000). The polymorphism of the 3'-UTR in the *CYP2A6* gene played an important role in *CYP2A6* mRNA stabilization and enzyme expression. Such polymorphisms have been described to influence the *in vivo* rate of nicotine elimination and possibly the cigarette consumption and risk of smoking-induced lung cancer (Wang *et al.* 2006). Although we are not going to conclude that the 'C' allele of the E-cadherin gene 3'-UTR polymorphism is the direct cause of endometriosis, we can indicate that there is an association. These findings suggest that the 3'-UTR C $\rightarrow$ T polymorphism may play a role in the etiology of endometriosis.

The etiology of endometriosis is uncertain, but implantation of viable endometrium refluxed into the peritoneal cavity during menstruation is a major theory (Thomas 1993). Since retrograde menstruation is a very common

phenomenon among women of reproductive age, there must be other factors that contribute to the pathophysiology and/or the pathogenesis of endometriosis. Genetic predisposition is believed to play a significant role in the establishment and maintenance of endometriosis. Whether the differential expression of E-cadherin between eutopic endometrium and ectopic biopsies of endometriosis patients and those of unaffected women is related to genetic factors that are associated with the risk for developing endometriosis is important for understanding the mechanism of endometriosis.

To the best of our knowledge, this is the first study to look for an association between the polymorphisms of the E-cadherin gene and the risk of developing endometriosis. Our study results suggest that certain genetic polymorphisms of the E-cadherin gene seem to be correlated with a risk of developing endometriosis. Further, larger population-based studies as well as functional evaluation of the variants are warranted to confirm our findings.

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