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A Functional Promoter Polymorphism in *NFKB1* Increases Susceptibility to Endometriosis

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Numerous proinflammatory cytokines, such as TNFα and IL-6, which are nuclear factor κB (NF-κB) target genes, have been shown to promote proliferation in endometriotic cells, and several other genes involved in promoting growth are also NF-κB target genes. The aim of this study was to investigate whether the functional insertion/deletion polymorphism (−94 insertion/deletion ATTG) in the promoter of nuclear factor κB gene (*NFKB1*) is associated with susceptibility to endometriosis. Polymerase chain reaction–polyacrylamide gel electrophoresis method was used to genotype the *NFKB1* −94 insertion/deletion ATTG polymorphism in 206 women with endometriosis and 365 ethnicity-matched healthy control women. The genotyping method was confirmed by the DNA sequencing analysis. Genotype at the −94 insertion/deletion ATTG polymorphism in the *NFKB1* promoter was in Hardy–Weinberg equilibrium in either case or control subjects. The frequency of the $ATTG_2/ATTG_2$ genotype and $ATTG_2$ allele in the endometriosis was significantly higher than that of control subjects (59.7% vs. 37%, odds ratio = 3.069, $p < 0.001$ for $ATTG_2/ATTG_2$ genotype; 75.2% vs. 59.7%, odds ratio = 2.049, $p < 0.001$ for $ATTG_2$ allele), indicating that the −94 insertion/deletion ATTG polymorphism in the *NFKB1* promoter was associated with endometriosis. This study suggests that the functional −94 insertion/deletion ATTG polymorphism in the promoter of *NFKB1* is associated with an increased risk for endometriosis.

Introduction

Endometriosis, defined as the presence of endometrial glands and stroma outside the uterine cavity, is a benign gynecologic disease affecting the eugenesis of 5–10% reproductive-aged women (The Practice Committee of the American Society for Reproductive Medicine, 2004). Although symptoms vary, they commonly included dysmenorrhea, dyspareunia, noncyclic pelvic pain, and infertility. Peritoneal inflammation, fibrosis, and the formation of adhesions and ovarian cysts are the main pathological processes associated with endometriosis. However, the underlying pathologic mechanism of this disease is still enigmatic to date. The transplantation theory, by which endometrial cells can implant and develop in ectopic locations, is the most widely accepted one for the explanation of endometriosis.

Endometriosis is a well-recognized estrogen-dependent disease. The medical treatment for endometriosis has focused on the hormonal alteration of the menstrual cycle with a major goal to produce a pseudo-pregnancy, pseudomenopause, or chronic anovulation, creating an acyclic, hypoestrogenic environment (Olive and Pritts, 2001; Kitawaki *et al.*, 2002). Recent large-scale gene expression profiling studies have revealed that many genes are dysregulated in endometriosis (Matsuzaki *et al.*, 2005; Wu *et al.*, 2006). Accumulating evidence supports that immunologic factors and inflammatory response factors play crucial roles in the pathogenesis of endometriosis, and endometriosis is associated with systemic subclinical inflammation, which is thought to be responsible for pain (Lebovic *et al.*, 2001; Wu and Ho, 2003; Thomson and Redwine, 2005; Agic *et al.*, 2006). In ectopic endometrium, increased production of proinflammatory cytokines and chemokines has been observed, affecting various aspects of reproduction in women with endometriosis and causing subfertility (Hornung *et al.*, 2001; Halis and Arici, 2004). Numerous proinflammatory cytokines, such as TNFα and IL-6, which are nuclear factor κB (NF-κB) target genes, have been shown to promote proliferation in endometriotic cells, and several other genes involved in promoting growth are also NF-κB target genes (Iwabe *et al.*, 2000; Guo, 2007).

NF-κB is a major transcription regulator of immune response, apoptosis, and cell-growth control genes, and there are five members of the NF-κB family in mammals:

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p50/p105, p65/RelA, c-Rel, RelB, and p52/p100. Although many dimeric forms of NF-κB have been detected, the major form of NF-κB is a heterodimer of the p50 and p65/RelA subunits, encoded by the *NFKB1* and *RelA* genes, respectively (Chen *et al.*, 1999). Several lines of investigation suggest that NF-κB could promote and maintain endometriosis, and, interestingly, all existing and nearly all investigational medications for endometriosis appear to act through suppression of NF-κB activation (Guo, 2007). In a recent study investigating the role of the NF-κB pathway on gene expression in the eutopic endometrium in endometriosis, molecular alterations were observed during the late secretory phase in eutopic endometrium from endometriosis patients, suggesting that NF-κB could be an important factor in endometriosis etiology (Ponce *et al.*, 2009).

A common insertion/deletion polymorphism (−94 insertion/deletion ATTG, rs28362491) located between two putative key promoter regulatory elements in the *NFKB1* gene was identified, which seems to be the first potential functional *NFKB1* genetic variation. The presence of a 4 base pair (bp) deletion resulted in the loss of binding to nuclear proteins, leading to reduced promoter activity (Karban *et al.*, 2004). A research has shown that the deletion ($ATTG_1$ allele) was associated with an increased risk for an inflammatory intestinal disorder (ulcerative colitis), but data from subsequent other studies were inconsistent (Karban *et al.*, 2004; Oliver *et al.*, 2005; Rueda *et al.*, 2006). It has also been reported that this polymorphism is associated with psoriasis, nasopharyngeal carcinoma, dilated cardiomyopathy, and oral squamous cell carcinoma (Lin *et al.*, 2006; Li *et al.*, 2008; Zhou *et al.*, 2009a, 2009b). However, its association with endometriosis is still unclear. The main goal of the present investigation was to determine the possible susceptibility of *NFKB1* −94 insertion/deletion ATTG polymorphism on the occurrence of endometriosis.

Materials and Methods

Subjects

This study was approved by the hospital ethics committee and all subjects gave written informed consent to participate. Two hundred and six women aged 18–58 years (mean ± standard deviation, 39.11 ± 7.02) with endometriosis diagnosed by laparotomy or laparoscopy between July 2007 and March 2009 were enrolled into the present study at the Second University Hospital of Sichuan University. The control group consisted of 365 healthy subjects aged 23–65 years (mean ± standard deviation, 36.99 ± 8.74) from two sources: hospital and community. The hospital control subjects were early-pregnancy women recruited from the Second University Hospital of Sichuan University. The community control group consisted of 192 healthy subjects from a routine health survey in the same hospital, and subjects with any personal or family history of endometriosis or other serious disease were intentionally excluded. All subjects were from the Han population living in Sichuan province of southwest China.

Genotyping

Genomic DNA of each individual was extracted from 200 μL EDTA-anticoagulated peripheral blood samples by a DNA isolation kit from Bioteke (Peking, China), and the procedure was performed according to the manufacturer's instructions. The polymerase chain reaction (PCR)–polyacrylamide gel electrophoresis method was used to genotype the −94 insertion/deletion ATTG polymorphisms of *NFKB1* (Zhou *et al.*, 2009a). In brief, the primer sequences were 5′-TGGA CCGCATGACTCTATCA-3′ (forward) and 5′-GGCTCTGGC TTCCTAGCAG-3′ (reverse), and DNA fragments containing the polymorphism were amplified in a total volume of 25 μL, including 2.5 μL 10× PCR buffer, 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 μM each primer, 100 ng of genomic DNA, and 1 U of *Taq* DNA polymerase. The PCR conditions were 94°C for 4 min, followed by 32 cycles of 30 s at 94°C, 30 s at 64°C, and 30 s at 72°C, with a final elongation at 72°C for 10 min. Three microliters of PCR products was separated by a 6% polyacrylamide gel and staining with 1.5 g/L argent nitrate. Allele $ATTG_1$ yielded a 154 bp band and allele $ATTG_2$ yielded a 158 bp band. The genotypes were confirmed by the DNA sequencing analysis (BigDye®Terminator v3.1 Cycle Sequencing Kits; Applied Biosystems, Foster City, CA). About 20% of the samples were randomly selected to perform the repeated assays, and the results were 100% concordant.

Statistical analysis

Allelic and genotype frequencies of the *NFKB1* gene −94 insertion/deletion ATTG polymorphism were obtained by directed counting, and Hardy–Weinberg equilibrium was evaluated by chi-square test. All data analyses were carried out using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). Odds ratio (OR) and respective 95% confidence intervals (CIs) were reported to evaluate the effects of any difference between allelic and genotype distribution. Probability values of 0.05 or less were regarded as statistically significant in endometriosis patients compared with control subjects.

Results

Three genotypes of the *NFKB1* −94 insertion/deletion ATTG polymorphism ($ATTG_1/ATTG_1$, $ATTG_1/ATTG_2$, and $ATTG_2/ATTG_2$) were successfully identified. Genotype distributions had no deviation from Hardy–Weinberg equilibrium both in patients and control subjects. The distribution of the *NFKB1* −94 insertion/deletion ATTG polymorphism among all patients with endometriosis and control subjects is shown in Table 1. The overall genotype frequency of endometriosis patients was significantly different from that of control subjects. The frequency for $ATTG_2/ATTG_2$ genotype was overrepresented in endometriosis patients ($p = 0.001$, OR = 2.844, 95% CI = 1.483–5.455 for patients vs. hospital control comparison; $p = 8.92E\text{-}007$, OR = 3.284, 95% CI = 1.746–6.175 for patients vs. community control comparison; and $p = 7.93E\text{-}007$, OR = 3.069, 95% CI = 1.740–5.412 for patients vs. all control comparison). The frequency of the $ATTG_2$ allele in endometriosis patients was significantly higher than that in control subjects (75.2%, 60.7%, 58.9%, and 59.7%, in endometriosis patients, hospital control, community control, and all control subjects), and $p = 1.74E\text{-}005$, OR = 1.968, 95% CI = 1.442–2.686 for patients vs. hospital control comparison; $p = 8.52E\text{-}007$, OR = 2.125, 95% CI = 1.571–2.875 for patients versus community control comparison; $p = 1.24E\text{-}007$, OR = 2.049, 95% CI = 1.567–2.680 for patients versus all

Table 1. Distribution of the −94 Insertion/Deletion ATTG Polymorphism in the *NFKB1* Gene in Endometriosis Patients and Control Subjects

	Patients, n = *206*	*Hospital control subjects*			*Community control*			*All control subjects*		
		n = *173*	*OR*	p-*Value*	n = *192*	*OR*	p-*Value*	n = *365*	*OR*	p-*Value*
NFKB1 −94 genotype (%)										
$ATTG_1/ATTG_1$	19 (9.2)	29 (16.8)			35 (18.2)			64 (17.5)		
$ATTG_1/ATTG_2$	64 (31.1)	78 (45.1)	1.252 (0.643–2.438)	NS	88 (45.8)	1.340 (0.703–2.553)	NS	166 (45.5)	1.299 (0.722–2.337)	NS
$ATTG_2/ATTG_2$	123 (59.7)	66 (38.2)	2.844 (1.483–5.455)	**0.001**	69 (35.9)	3.284 (1.746–6.175)	**8.92E-007**	135 (37.0)	3.069 (1.740–5.412)	**7.93E-007**
NFKB1 −94 allele (%)										
$ATTG_1$	102 (24.8)	136 (39.3)	1.968 (1.442–2.686)	**1.74E-005**	158 (41.1)	2.125 (1.571–2.875)	**8.52E-007**	294 (40.3)	2.049 (1.567–2.680)	**1.24E-007**
$ATTG_2$	310 (75.2)	210 (60.7)			226 (58.9)			436 (59.7)		

Boldfaced values indicate a significant difference at the 5% level.
NS, not significant; OR, odds ratio.

control comparison. No significant differences were found in the genotype and allele distributions between hospital control and community control subjects.

Discussion

Endometriosis, caused by the interaction between genetic and environmental factors, is commonly regarded as a complex disorder that often recurs and has a major impact on women's health. Higher rates of endometriosis are found among the relatives of endometriosis patients compared with that of control subjects in both hospital and community samples (Simpson and Bischoff, 2002; Stefansson *et al.*, 2002; Montgomery *et al.*, 2008). In a study of the genetic influence on endometriosis risk in an Australian twin sample, results show that the risk ratio of affected versus population prevalence was 3.58 for monozygotic twins and 2.32 for dizygotic twins (Treloar *et al.*, 1999). A number of different studies support the contribution of genetic factors in endometriosis susceptibility (Zondervan *et al.*, 2001; Simpson and Bischoff, 2002; Stefansson *et al.*, 2002; Montgomery *et al.*, 2008; Tempfer *et al.*, 2009).

In the present case–control study, we have identified a significant association between the −94 insertion/deletion ATTG polymorphism in *NFKB1* promoter and the risk of endometriosis. Individuals homozygous for $ATTG_2$ have 3.069-fold risk to develop endometriosis compared with that homozygous for $ATTG_1$ ($p < 0.001$). Individuals with $ATTG_2$ alleletype have 2.049-fold risk for endometriosis compared with those carrying $ATTG_1$ alleletype ($p < 0.001$). To the best of our knowledge, this is the first molecular epidemiologic study to associate the *NFKB1* gene polymorphism with the risk of endometriosis development.

The −94 insertion/deletion ATTG polymorphism was identified in a study that sequenced the *NFKB1* promoter in 10 inflammatory bowel disease patients and 2 control subjects, and the $ATTG_1$ allele was more frequent in patients with ulcerative colitis than in control subjects in the following study. The *in vitro* promoter expression studies suggest that the $ATTG_1$ allele may result in relatively decreased *NFKB1* message and hence decreased p50/p105 NF-κB protein production (Karban *et al.*, 2004). Given the considerable important role of NF-κB pathway involved in initiation and progression of pathogenesis in disease, the association between the −94 insertion/deletion ATTG polymorphism in *NFKB1* promoter and different disease has been widely studied in different populations (Sun and Zhang, 2007; Zhou *et al.*, 2009a, 2009b).

NF-κB plays an important role in complicated pathogenetic regulation of apoptosis, differentiation, and senescence in addition to tumorigenesis (Criswell and Arteaga, 2007; Yang *et al.*, 2007). The transcription of many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis, and apoptosis is regulated by NF-κB. In most cells, inactive NF-κB complexes reside in the cytoplasm via their noncovalent interaction with inhibitory proteins known as IκBs, and the phosphorylation IκBs is mediated by a multimeric complex called IκB kinase complex (Chen *et al.*, 1996). In endometriosis, stimulation of proinflammatory cytokines such as TNFα and IL-1β would induce NF-κB activation, which, in turn, would stimulate further production of proinflammatory cytokines, resulting in the endometriotic

cells in a state of constant NF-κB activation, and a cascade of downstream changes (Guo, 2007).

Many genes that are dysregulated in endometriosis are NF-κB target genes. *C-myc*, a protooncogene that has been shown to be regulated by NF-κB, is activated in ectopic and eutopic endometrium from women with endometriosis, suggesting that transformation of cells from normal to endometriotic phenotype may be mediated in part through NF-κB activation (Schneider *et al.*, 1998; Johnson *et al.*, 2005; Guo, 2007). Many lines of evidence suggest that endometriotic cells have reduced apoptosis (Beliard *et al.*, 2004). *Bcl-2* and survivin, two anti-apoptotic genes regulated by NF-κB, are upregulated in endometriotic cells (Jones *et al.*, 1998; Ueda *et al.*, 2002). A prominent feature of endometriotic cells that sets them apart from eutopic endometrial cells is their invasiveness (Gaetje *et al.*, 1995). In endometriosis, expression of urinary plasminogen activator, matrix metalloproteinases, and IL-8, which are all NF-κB regulated and involved in inducing invasiveness, is dysregulated (Fernandez-Shaw *et al.*, 1995; Chung *et al.*, 2001; Darai *et al.*, 2003). Constant NF-κB activation results in dysregulation of urinary plasminogen activator, matrix metalloproteinases, and IL-8, rendering endometriotic cells invasive (Guo, 2007). Many factors that are NF-κB regulated and involved in angiogenesis (angiogenesis is one of key components in the pathogenesis of endometriosis) have been identified, including IL-8, monocyte chemoattractant protein 1, TNFα, and vascular endothelial growth factor (VEGF) (Levine *et al.*, 2003; Guo, 2007). NF-κB also regulates several genes involved in inflammation and oxidative stress. Once NF-κB is activated, these genes are subsequently induced, conferring anti-apoptosis, proliferation, oxidative stress, angiogenesis, invasion, and increased production of proinflammatory cytokines and chemokines, which, altogether, lead to pelvic pain and subfertility (Guo, 2007).

In conclusion, our study showed for the first time the association of the *NFKB1* −94 insertion/deletion ATTG polymorphism with the risk of endometriosis development. The $ATTG_2$ allele of the *NFKB1* −94 insertion/deletion ATTG polymorphism may be correlated with the risk in women of developing endometriosis. However, additional studies in a larger number of endometriosis patients and in different populations could help to establish the true significance of this association.

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Disclosure Statement

No competing financial interests exist.

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