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Research paper

A functional promoter polymorphism in interleukin 12B gene is associated with an increased risk of ovarian endometriosis

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ABSTRACT

Objective: To explore the role of functional genetic variant of *IL12B* in the pathogenesis of ovarian endometriosis.
Study design: This study included 815 patients with ovarian endometriosis and 788 women in the control group. Two polymorphisms were genotyped by a method of polymerase chain reaction and ligase detection reaction. To assess the biological significance of polymorphisms, we detected the level of IL12p40 protein expression in patients' eutopic endometrial tissues using quantitative real-time polymerase chain reaction (qRT-PCR).
Result(s): Compared with the CTCTAA/CTCTAA genotype, the GC/GC and GC/CTCTAA genotypes of rs17860508 could significantly increase the risk of ovarian endometriosis development (OR = 1.58, 95% CI = 1.19–2.09; OR = 1.37, 95% CI = 1.08–1.74, respectively). Moreover, the IL12B mRNA was expressed at significantly higher levels in the eutopic endometrial tissue of patients with the GC/GC genotype than in patients with the CTCTAA/CTCTAA genotype. However, the rs3212227 polymorphism may not be associated with a risk of ovarian endometriosis.
Conclusion(s): The rs17860508 polymorphism in the *IL12B* promoter region may influence the risk of developing ovarian endometriosis by altering the endometrial expression of *IL12B* of in Northern Chinese women.

1. Introduction

Endometriosis is one of the most common gynaecological diseases and is characterized by the growth of endometrial glands and stroma outside the uterine cavity, such as in the ovaries, the pouch of Douglas and the peritoneal surface (Giudice & Kao, 2004). Its main symptoms include noncyclic pelvic pain, dysmenorrhea, dyspareunia and infertility, which greatly deteriorate quality of life (Laganà et al., 2017; Goldman & Cramer, 1990; Vitale et al., 2017). The most widely accepted theory to explain the pathogenesis of the disease is the Sampson theory, which suggests that the shed endometrial tissues reflux into the peritoneal cavity through the fallopian tubes and then implant, proliferate, and develop into endometriotic lesions (Sampson, 1927). Although up to 90% of women of reproductive age have retrograde menstruation, the reasons why some develop endometriosis remain unclear (Bartosik et al., 1986; Cramer & Missmer, 2002). The retrograde menstruation theory also does not completely explain the pathogenesis of endometriosis that develops at other sites (i.e. bladder endometriosis, caesarean scar endometriosis) (Leone et al., 2017; Saccardi et al., 2017; Burney & Giudice, 2012; Vetvicka et al., 2016; Laganà et al., 2017). A growing body of evidence suggests that impaired immunological clearance of endometrial fragments shed into the peritoneal cavity may facilitate the implantation of endometrial cells in ectopic locations and result in endometriosis (Viganò et al., 1991; Guo et al., 2016; Oosterlynck et al., 1992; Podgaec et al., 2007; Dmowski et al., 1994).

The impaired peritoneal immune response of patients with endometriosis has been postulated to be related to decreased cytokine secretion by T helper (Th) lymphocytes and reduced cytotoxic activity of natural killer (NK) cells, which together may facilitate the establishment of peritoneal endometriosis (Oosterlynck et al., 1991; Ho et al., 1995; Ho et al., 1996; Szyllo et al., 2003). Interleukin12 (IL12), a key molecule in the immune system, participates in the conversion of primordial T cells into a Th1 pattern (Trinchieri, 1993) and regulates the activity of antigen-presenting and NK cells (D'Andrea et al., 1992). Unlike most other cytokines, IL12 is a heterodimeric cytokine comprising p35 and p40 subunits encoded by separate genes (*IL12A* and

Abbreviations: IL12B, interleukin 12B; Th lymphocytes, T helper lymphocytes; NK cells, natural killer cells; IL12, interleukin12; 3′ UTR, 3'untranslated region; EDTA, ethylenediaminetetraacetic acid; cDNA, complementary DNA; qRT-PCR, quantitative real-time polymerase chain reaction; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; CI, confidence interval; ANOVA, one-way analysis of variance; PBMC, peripheral blood mononuclear cells; QR, quartile range

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IL12B, respectively). *IL12B* encodes the IL12p40 protein, which not only is a component of bioactive IL12 but also has intrinsic functional activity. The most widely appreciated function of IL12p40 is to negatively regulate IL12 by competitively binding to the IL12 receptor (Mattner et al., 1993; Ling et al., 1995). Therefore, *IL12B* may be involved in the pathogenesis of endometriosis.

The rs17860508 CTCTAA/GC and rs3212227A/C polymorphisms, two potential functional genetic variants located in the promoter and 3'untranslated region (UTR) of *IL12B*, respectively, have been reported to be associated with altered levels of gene transcription and mRNA stability in vitro (Morahan et al., 2002; Khoo et al., 2004; Hirota et al., 2005). Further evidence of the functional importance of these two variants includes significant associations with immune-related diseases and cancers (Yanagihori et al., 2006; Naka et al., 2009; Tao et al., 2012; Walsh et al., 2011). Based on the role of IL12p40 in endometriosis (Mazzeo et al., 1998), we hypothesized that these two polymorphisms may contribute to the risk of developing ovarian endometriosis. We conducted a two-part study to test this hypothesis i) We investigated the association between rs17860508 and rs3212227 polymorphisms and the risk of developing ovarian endometriosis in Northern Chinese women and ii) analysed the level of the *IL12B* mRNA expression in eutopic endometrial tissues from patients with ovarian endometriosis who carry different genotypes for the two polymorphisms.

2. Materials and methods

2.1. Study participants

A case-control study design was used that included 815 women with ovarian endometriosis and 788 women without ovarian endometriosis who served as controls. A large part of the sample population has been used in our previous studies (Kang et al., 2013; Kang et al., 2014; Kang et al., 2010). All patients with ovarian endometriosis underwent laparoscopy or laparotomy at the Fourth Affiliated Hospital of Hebei Medical University between 2004 and March 2016, and they all had a histologically confirmed diagnosis. Patients were diagnosed with moderate or severe endometriosis (stage III–IV) according to the revised American Fertility Society classification system (1997). The mean age and menarche age of the patients was 35.85 ± 7.64 and 14.54 ± 1.55 years, respectively. General detailed information for each patient was recorded in her medical chart.

The control group was randomly selected from women of reproductive age without any malignant disease, autoimmune disease or endometriosis, which was confirmed either by surgical exploration or ultrasound examinations. The control group consisted of women who received a health examination (n = 248), hydrosalpinx (n = 250) or hysterectomy for dysfunctional uterine bleeding (n = 290). The mean age and menarche age of the controls was 36.04 ± 8.53 and 14.49 ± 1.58 years, respectively. The general information for the controls was collected from their medical charts.

Eutopic endometrial tissues were collected from 45 patients with an average age of 39.83 ± 5.45(30–49) years who received laparoscopy or laparotomy due to ovarian endometriosis. The patients' age at menarche onset was 13.73 ± 1.37 years and parity was 1.13 ± 0.46 births. All women with endometriosis presented with a regular menstrual cycle (the cycle length was approximately 25–32 days). The cycle stage was estimated according to the date of the last menstrual phase or by histological evaluation (Noyes et al., 1975). All samples were exclusively collected in the secretory phase of the menstrual cycle by curette. In the 6 months prior to surgery, none of the study subjects received any hormone therapy. All tissue samples were stored in RNA later solution (Ambion, Carlsbad, New Mexico, USA) immediately after surgical dissection and were subsequently stored in a −20 °C freezer.

All subjects were women of Han ethnicity in Northern China. This study was approved by the ethics committee of the Hebei Obstetrics and Gynecology Institute and informed consent was obtained from all recruited subjects.

2.2. DNA extraction

Venous blood (5 mL) from each subject was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA), and the tubes were stored at 4 °C. Genomic DNA was extracted within 1 week after sampling using proteinase K (Merck, Darmstadt, Hesse, Germany) digestion followed by a salting-out procedure performed according to the methods of Miller et al. (Miller et al., 1988).

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the eutopic endometrial tissues of women with endometriosis using Trizol reagent (Generay, Shanghai, China) as recommended by the manufacturer's instructions. The concentration of the purified RNA was determined with a NanoDrop 2000C spectrophotometer at 260/280 nm (Thermo Fisher Scientific, Massachusetts, USA). The total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's directions. The cDNA was subsequently stored in a −20 °C freezer.

2.4. Genotyping of IL12B polymorphisms

The genotypes of two polymorphisms (rs17860508 and rs3212227) were determined by Shanghai Generay Biotech Co., Ltd. (http://www.generay.com.cn) using the polymerase chain reaction/ligase detection reaction (PCR-LDR) method. The primers and lengths of the PCR products are summarized in Table 1. Products were analysed using an ABI 3730XL DNA sequencer (Applied Biosystems) after the PCR ligase detection reactions. In addition, representative PCR products were subjected to direct DNA sequencing to confirm the accuracy of this method.

2.5. QRT-PCR analysis of IL12B mRNA expression levels

QRT-PCR was carried out using the QuantiNova SYBR Green PCR kit (Qiagen, Shanghai, China) with the two-step qRT-PCR System in triplicate. The qRT-PCR primers for *IL12B* were as follows: forward primer 5′-CCCTGACATTCTGC GTTCA-3′ and reverse primer 5′- AGGTCTTGT CCGT GAAGACTCTA-3′. GAPDH mRNA was used as an internal control, and it was amplified with the forward primer 5′-ACCACAGTCCATGC CATCAC-3′ and the reverse primer 5′-TCCACCACCCT GTTGCTGTA-3′. Amplification reactions were executed in a 20 μL reaction volume containing 1.4 μL of forward primers, 1.4 μL of reverse primers, 10 μL of Master mix, 0.1 μL of ROX,1 μL of cDNA and 6.1 μL of water. The cycling conditions were set at 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 25 s. Melting curves were generated after each run to verify the amplification of the desired amplicons, which was determined by the presence of a single specific peak and the absence of primer dimers. The $2^{-\Delta CT}$ algorithm was used to calculate the expression of individual *IL-12B* relative to expression of *GAPDH*.

Table 1
Primers sequences and products length for the two polymorphisms in IL12B gene.

Polymorphisms	Primers	Product length
rs17860508	5′-CGGCTCCTGATTCCATTTTTGTAC -3′ (F) 5′-GCTTCTGGGAGGGATGGGAGTATA -3′ (R)	173 bp
rs3212227	5′-CGCGAACGAAGGAAGTGAGTCA -3′ (F) 5′-GCCGCCAGCACCCGAAAC -3′ (R)	179 bp