The tumor necrosis factor-α promoter – 1031C polymorphism is associated with decreased risk of endometriosis in a Japanese population

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BACKGROUND: Tumor necrosis factor-α (TNF-α) is a multifunctional proinflammatory cytokine, associated with various inflammatory and autoimmune diseases. Elevated TNF-α levels in peritoneal fluid have been reported in women with endometriosis, suggesting that TNF-α may be involved in the development of endometriosis. In this study, we investigated the possible association between endometriosis and the TNF-α gene promoter polymorphisms –238G/A, –308G/A, –857C/T, –863C/A and –1031T/C in a Japanese population. METHODS: We compared the distribution of the –238G/A, –308G/A, –857C/T, –863C/A and –1031T/C polymorphisms in the promoter region of TNF-α in 130 endometriosis cases and 185 controls using PCR–RFLP analysis. RESULTS: The allele frequencies of –238A, –308A, –857T, –863A and –1031C in controls were 2.0%, 1.3%, 19.4%, 17.0% and 18.6%, and in the cases 1.1%, 0.3%, 19.6%, 18.6% and 13.6%, respectively. No significant differences in frequencies were found between the crude endometriosis cases and controls. However, when the endometriosis group was divided into a subgroup of women with stage IV disease only, the frequency of the –1031C allele was significantly lower in this subgroup than controls. CONCLUSIONS: The variability of the –1031T/C polymorphism of the TNF-α gene may be associated with susceptibility to (AUTHOR: as meant?) endometriosis.

Key words: endometriosis/gene polymorphism/PCR/restriction fragment length polymorphism/TNF-α

Introduction

Endometriosis is characterized by the growth of endometrial tissue implants in ectopic sites (Olive et al., 1993; Libvoic et al., 2001). Recently, an increasing body of evidence that endometriosis has a genetic basis (Kennedy et al., 1995, 1997, 1998; Hadfield et al., 1997) has stimulated research into the genes involved in the pathogenesis of this disease. However, despite significant efforts, the precise genetic mechanisms leading to endometriosis still remain unknown.

Tumor necrosis factor alpha (TNF-α) gene is one of many genes previously implicated, based on the finding that elevated TNF-α levels in peritoneal fluid have been associated with up-regulated TNF-α production in peritoneal macrophages and peripheral monocytes of women with endometriosis (Keenan et al., 1995; Braun et al., 1996). It has been suggested that a peritoneal immuno-surveillance network involving different cell types may sub-serve this role, with the consequence that the immune system appears critical for the development of this disease (Braun and Dmowski, 1998; Libvoic et al., 2001). Although the functional role of TNF-α in endometrial tissue is unknown, it is conceivable that TNF-α may be associated with the susceptibility to endometriosis.

TNF-α is a multifunctional proinflammatory cytokine that plays an important role in the initiation and regulation of immune responses. It activates inflammatory leukocytes and stimulates macrophages to produce other cytokines, such as interleukin (IL)-1, IL-6 and TNF-α itself (Lee et al., 2002). Upon interaction of TNF-α with one of its receptors, TNF receptor 1 or TNF receptor 2 (Beutler et al., 1994), a variety of responses are elicited which affect the regulation of a large number of genes (Beutler, 1995).

TNF-α is also a significant source of genetic variability. Sequence polymorphisms, especially in the promoter region, have been identified that could play a part in the transcriptional regulation of the TNF-α gene. A G to A substitution at position –238 (D’Alfonso et al., 1994), a G to A substitution at position –308 (Wilson et al., 1992), a C to T substitution at position –857 (Hermann et al., 1998), a C to A substitution at position –863 (Ugliarolo et al., 1998) and a T to C
substitution at position −1031 (Higuchi et al., 1998) have been described in the proximal promoter of the TNF-α gene.

Two studies (Lee et al., 2002; Wieser et al., 2002) have previously examined the association between endometriosis and TNF-α gene promoter polymorphisms at −238G/A and −308G/A in Caucasian and Korean populations, but no evidence of association was found. However, there has been no published study examining the association between endometriosis and the −857, −863 and −1031 polymorphisms in the promoter region of TNF-α gene. In this study, we investigated whether these five common polymorphisms in the promoter region of the TNF-α gene are associated with endometriosis in a Japanese population.

Materials and methods

Subjects

The Ethics Review Committee on Genetic and Genomic Research of Kobe University Graduate School of Medicine approved the design of this study. Informed consent was obtained from all the women involved in the study, as well as the parents of newborn female infants who provided umbilical cord blood for use as population controls. The patient group consisted of 130 unrelated, ethnically Japanese women with endometriosis, confirmed by examining their operative records and histological findings. Patients were excluded from the study if the operative records were unavailable or if there was any doubt about the diagnosis. All patients were surgically confirmed to have stage I–IV of disease according to the revised American Society for Reproductive Medicine classification of endometriosis (1996): minimal (stage I) = 3 cases (2.3%), mild (stage II) = 12 cases (9.2%), moderate (stage III) = 25 cases (19.2%) and severe (stage IV) = 90 cases (69.3%). Samples of umbilical cord blood, obtained from 185 female neonates born at the Hayashi Clinic in Kobe, Japan were used as Japanese population controls. All the babies were delivered between 37 and 41 gestational weeks and had a birth weight >2500 g with an Apgar score of 9 or more at 5 min. Those with non-Japanese ethnic/racial parents were excluded, as were those whose mothers had medical conditions, such as pre-eclampsia, hypertension and diabetes mellitus. These controls were chosen as they were considered to be a reasonable population-based control group. Since minimal-mild endometriosis is commonly found in asymptomatic women and may represent a normal physiological process (Zondervan et al., 2002), we performed a separate analysis on patients with stage IV disease only in the present study.

Genotyping

Genomic DNA was extracted from EDTA anti-coagulated whole blood using the Wizard DNA purification kit (Promega, Madison, WI). The −238, −308, −857, −863 and −1031 polymorphisms in the promoter region of TNF-α gene were determined by using PCR–RFLP analysis, as previously described (Skoog et al., 1999; Van Heel et al., 2002). Genotyping for the −238G/A polymorphism was performed using a PCR fragment amplified by the forward primer 5'-AAACAGACCCACACCTTGGTCT and the reverse primer 5'-CTCACACCTTGTTCCTTTCCCGAAGTC. The reverse primer contained two nucleotide mismatches, which made it possible to use the restriction enzyme BamHI for the detection of the −308G/A polymorphism. Genotyping for the −857C/T and −863C/A polymorphisms was performed using the forward primer 5'-GGCTCTGAGGAATGGGTTAAC and the reverse primers 5'-CCTCTACAGGCGCTTCTAC and 5'-CTCACATGGCCTGTTACCAG, respectively. The two reverse primers each contain a nucleotide mismatch, which made it possible to detect both polymorphisms using the restriction enzyme HpaCH4IV. It is noteworthy that the reverse primer for the −863C/A polymorphism overlaps with the −857C/T polymorphism and contains the wild-type C nucleotide at the −857 position. The −1031T/C polymorphism was evaluated using the forward primer 5'-TATGTTGATGGACTCACCAGGT and the reverse primer 5'-CCTCTACATGGCCTGGTCTT, followed by digestion with the restriction enzyme BbsI.

The conditions for the genotyping were: PCR in a 20 μl reaction mixture containing 20 ng of genomic DNA, 10 pmol of each primer, 250 μM of each dNTP and 1.0 units Taq gold DNA polymerase. The concentration of MgCl2 varied between the PCR reactions for the different polymorphisms and was 1.5 mM for the −238 locus, 1.0 mM for −308, 2.0 mM for −857, 1.25 mM for −863 and 1.0 mM for −1031. The PCR was conducted with ABI 9700 thermocycler (PE Applied Biosystem, Foster City, CA) using the following thermal profile: an initial denaturing cycle of 96°C for 12 min; 35 cycles of denaturing at 94°C for 30 sec, annealing at 59°C for 1 min, and extension at 72°C for 2 min; and a final cycle of 72°C for 2 min. Digestions with the appropriate restriction enzymes were performed as described by the manufacturer (New England Biolabs, Beverly, MA) at 37°C for 16 h. DNA fragments were subjected to electrophoresis in a 4% agarose gel in the case of −238, −308, −857, −863 and a 2% agarose gel in the case of −1031. Gel was stained with ethidium bromide (0.1 μg/ml) and visualized by ultraviolet illumination.

Statistical methods

Genotypic distributions were examined for significant departure from the Hardy–Weinberg equilibrium by a goodness of fit χ2 test. χ2 test was used to examine differences in the proportions of genotypes of the five polymorphisms between endometriosis cases and controls. The Fisher correction was applied when appropriate. Odds ratios (OR) and 95% confidence intervals (CI) were used to compare categorical variables. Cases were also divided into a subgroup containing women with stage IV disease only, and the distribution of the five polymorphisms in this subgroup was analyzed separately.

Results

Genotyping of the five polymorphisms −238, −308, −857, −863 and −1031 was successful in 129/130 (99.2%), 126/130 (96.9%), 130/130 (100%), 129/130 (99.2%), 129/130 (99.2%) of the endometriosis cases and 175/185 (96.9%), 179/185 (96.7%), 183/185 (98.9%), 180/185 (97.2%), 180/185 (97.2%), of the controls. Allele and genotype frequencies were all in Hardy–Weinberg equilibrium in each group. The genotypes and allele frequencies of the polymorphisms −238G/A, −308G/A, −857C/T, −863C/A and −1031T/C in endometriosis cases and controls are shown in Table I. The allele frequencies of the polymorphisms −238A and −308A were 1.1% and 0.3% in the endometriosis cases and 2.0% and 1.3% in controls, respectively. Given the low frequencies of these alleles in a Japanese population, the sample number in this disease was too small to assess
Allele frequencies were higher than significant difference was observed in the frequency of the a subgroup of 90 women with stage IV disease only, a significance was observed in the frequency of the controls. Taken together, no significant difference was observed in the frequencies of the polymorphisms in a Japanese population, the genotype frequencies in the crude endometriosis cases were similar to those in the controls. Thus, allele frequencies of the other polymorphisms were comparable to population controls in other studies (Negoro et al., 1999; Skoog et al., 1999; Escobar-Morreale et al., 2001; Table II). Although these three polymorphisms were very polymorphic and allele frequencies were higher than −238 and −308 polymorphisms in the crude endometriosis cases were similar to those in the controls. Taken together, no significant difference was observed in the frequencies of the five polymorphisms between the crude endometriosis cases and controls in our study.

However, when the endometriosis cases were divided into a subgroup of 90 women with stage IV disease only, a significant difference was observed in the frequency of the −1031T/C polymorphism between this subgroup and controls (Table I). The frequency of the −1031C allele was significantly lower in stage IV endometriosis cases than controls (P = 0.04; χ² = 4.124; OR = 1.75; 95% CI = 1.019–3.01). Allele frequencies of the polymorphisms −857T, −863A and −1031C were 17.7%, 17.6% and 11.7% in this subgroup.

### Discussion

In this study, we have investigated the association of endometriosis with five common polymorphisms in the 5′-flanking region of the TNF-α gene in a Japanese population by using PCR–RFLP analysis. We observed no significant differences in the frequencies of the −238, −308, −857, −863 and −1031 promoter polymorphisms of the TNF-α gene between the crude endometriosis patients and controls in a Japanese population (Table I). Since minimal/mild endometriosis is commonly found in asymptomatic women and may represent a normal physiological process, we also performed a separate analysis on patients with the most severe disease in our study. Interestingly, when the endometriosis cases were divided into a subgroup of 90 women with stage IV disease only, a significant difference was observed in the frequency of the −1031C allele compared to the control group (P = 0.04; χ² = 4.124; OR = 1.75; 95% CI = 1.019–3.01).

### Table I. Genotype and allele frequencies of the TNF-α polymorphism among women with endometriosis and controls

<table>
<thead>
<tr>
<th>Position and disease</th>
<th>Genotypes (%)</th>
<th>P-value vs controls</th>
<th>Allele frequency (%)</th>
<th>P-value vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>−238</td>
<td>G/G G/A A/A</td>
<td></td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td>Endometriosis (n = 129)</td>
<td>127 (99.2) 1 (0.78) 1 (0.42)</td>
<td>0.70^a</td>
<td>255 (98.8) 3 (1.1)</td>
<td>0.40^a</td>
</tr>
<tr>
<td>Stage IV Endometriosis (n = 76)</td>
<td>76 (100) – –</td>
<td>–</td>
<td>152 (100) –</td>
<td>0.87^a</td>
</tr>
<tr>
<td>Controls (n = 157)</td>
<td>168 (96.0) 7 (4.0) –</td>
<td>–</td>
<td>343 (98.0) 7 (2.0)</td>
<td>–</td>
</tr>
</tbody>
</table>

### Table II. Comparison of the −1031 genotype and allele frequencies in this study and previously published studies (controls)

<table>
<thead>
<tr>
<th>Allele –1031 genotypes (%)</th>
<th>Allele frequency (%)</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T T/C C/C</td>
<td>T/C C/T T/T</td>
<td>T/T C/C</td>
</tr>
<tr>
<td>−1031</td>
<td>18 (65.1) 57 (31.7) 5 (2.8)</td>
<td>293 (81.4) 67 (18.6)</td>
</tr>
<tr>
<td></td>
<td>405 (70.4) 156 (27.1) 14 (2.4)</td>
<td>966 (84) 184 (16)</td>
</tr>
<tr>
<td></td>
<td>11 (45.8) 10 (41.6) 3 (12.5)</td>
<td>32 (84.0) 16 (16.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a versus controls.
^b χ² = 4.124; OR = 1.75; 95% CI = 1.019–3.01.
divided into a subgroup with stage IV disease only, a significant difference in the frequency of the −1031C polymorphism in the TNF-α gene promoter was noted between this group and controls \((P = 0.04; \text{Table I})\). This is the first study to demonstrate an association between severe endometriosis and the polymorphisms in the promoter region of the TNF-α gene. The association can be interpreted in three ways. First, the −1031C polymorphism may provide protection from the most severe endometriosis. Secondly, the positive association may result from linkage disequilibrium between another gene and this polymorphism. Thirdly, this finding could be the result of multiple statistical comparisons and no Bonferroni correction was applied.

A review of the literature highlighted the importance of several polymorphisms in the TNF-α gene region (Wilson et al., 1992). The polymorphisms at positions −238, −308, −857, −863 and −1031 have been associated with increased transcriptional activity and production of TNF-α in several studies (Wilson et al., 1997; Higuchi et al., 1998; Ahmad et al., 2003). The polymorphism at the −238 G/A has been associated with insulin resistance syndrome and obesity (Rasmussen et al., 2000). The −308 G/A polymorphism has been associated with various inflammatory and autoimmune diseases (Verjans et al., 1994; Cabrera et al., 1995; Mizuki et al., 1995; Wilson et al., 1995; Fong et al., 1996; Nadel et al., 1996). The −857, −863 and −1031 polymorphisms are associated with both increased luciferase activity and increased concanavalin-A stimulated TNF-α production from peripheral blood mononuclear cells (Higuchi et al., 1998). Associations between the three polymorphisms −857, −863, −1031 and immune-mediated diseases such as rheumatoid arthritis and Crohn’s disease have been reported (Wilson et al., 1992; Negoro et al., 1999). To date, two studies have investigated the TNF-α gene polymorphisms at −238G/A and −308G/A in patients with endometriosis in the Austrian and Korean populations, but failed to find any evidence of association. The frequencies of the −238A and −308A alleles in TNF-α varies significantly between ethnic groups, and it is rare in the Japanese population (<3%) (Sakao et al., 2001). Therefore, large sample populations are necessary to evaluate associations between the −238G/A and −308G/A polymorphisms and diseases in the Japanese population. At present, there is limited evidence showing that these allelic variants, at positions −238 and −308, are involved in the regulation of cytokine production (Wilson et al., 1997; Kaluza et al., 2000; Koch et al., 2000).

The −1031T/C polymorphism has recently been reported to be associated with Behcet’s disease (Ahmad et al., 2003), extra intestinal manifestations of Crohn’s disease including uveitis, erythema nodosum and large joint arthropathy (Orchard et al., 2002) and Crohn’s disease itself (Negoro et al., 1999). Negoro et al., (1999) and Ahmad et al., (2003) both reported an increased frequency of the −1031C allele in patients with Crohn’s disease and in patients with Behcet’s disease compared to controls. In contrast, a lower frequency of −1031C allele is reported in patients with hyperandrogenism and ulcerative colitis than in controls (Negoro et al., 1999; Escobar-Morreale et al., 2001). Our data are in agreement with the results of Escobar-Morreale et al., (2001) and Negoro et al., (1999) who showed a decreased frequency of the −1031C allele in these related diseases compared to controls, while the functional correlate of decreased −1031C allele in various diseases remain unclear. Further investigation is necessary to determine the functional significance of the TNF-α−1031C allele and how it interacts with the inflammatory dysregulation associated with endometriosis. In our study, a decreased frequency of −1031C allele is associated with severe endometriosis but not with less severe disease. The results imply that the C allele could provide protection from severe forms of endometriosis. It is striking that TNF-α promoter polymorphisms have been associated with the most severe manifestations of a number of other diseases (McGuire et al., 1994; Nadel et al., 1996; Knight et al., 1999).

The TNF gene is located on the short arm of chromosome 6, very close to the HLA class II locus. This region remains a major challenge because of the extensive linkage disequilibrium across this highly polymorphic region. Higuchi et al., (1998) investigated the transcriptional promoter activity of the −857T or −1031C−863A allele in response to concanavalin A stimulation, and reported that these activities were 1.7- or 2.0-fold higher than those of the dominant allele, respectively. They also reported that the −1031C allele is in linkage disequilibrium with HLA-DRB1*0901. Ishii et al., (2002) have examined the possible association between endometriosis and HLA-DR. They showed that the prevalence of the HLA-DRB1 *1403 allele was significantly higher in endometriosis patients than controls, suggesting that HLA might be involved in the etiology of endometriosis. However, they did not find an association between endometriosis and HLA-DRB1*0901. Taken together, it is possible that the low frequency of −1031C allele in endometriosis is not caused by linkage disequilibrium with HLA-DR, but the allele itself may independently protect women from the disease. Interestingly, Ahmed et al., (2003) reported that the association with TNF-1031C appeared to be independent of linkage disequilibrium with other polymorphic genes in the HLA region in Behcet’s disease. It remains incompletely determined whether the −1031T/C polymorphism of the TNF-α promoter is directly associated with susceptibility to endometriosis, or if it only represents a marker for some other closely linked susceptibility gene.

A relationship between the immune system and endometriosis was recognized almost 15 years ago, with the first description of TNF-α in peritoneal fluid of women with endometriosis (Eisermann et al., 1988). It has become clear since then that several molecular entities produced by cells in the immune system play a determining role in the establishment and maintenance of endometriosis (Bischof and Simpson, 2000; Harada et al., 2001; Nothnick, 2001; Vand en Linder, 2001; Braun et al., 2002). The most widely studied cytokines in that regard are interleukin-1 (IL-1), IL-6 and TNF-α. TNF-α is therefore a good candidate for studies related to the development and progression of endometriosis.

A genome-wide screen with micro-satellite markers testing for linkage in sister pairs affected with endometriosis is
already under way (Kennedy et al., 2001), but doubts remain about the power of this approach alone to identify genes implicated in the disease process—hence the need for studies of functional candidates. The hypothesis-driven investigation of functional candidate genes (i.e. those chosen on the basis of biological plausibility or known aberrant mechanisms in a disease) is a complementary approach to linkage analysis, which is hypothesis free, in the identification of susceptibility genes.

In conclusion, our data demonstrate a lower frequency of the −1031C polymorphism in the promoter region of the TNF-α gene in the most severe cases of endometriosis in a Japanese population, suggesting that the −1031C polymorphism may play some protective role from the progression of endometriosis. Although this model is biologically plausible, we recognize that our conclusions are based on relatively small numbers and will require verification from additional independent studies.

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