Genetic variation in tumour necrosis factor and lymphotoxin is not associated with endometriosis in an Australian sample

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BACKGROUND: Tumour necrosis factor (TNF) is a pleiotropic cytokine with a wide range of immunoregulatory effects. Variation in the promoter region of TNF and the neighbouring lymphotoxin alpha (LTA) gene might be associated with endometriosis.

METHODS: We examined the association between endometriosis and common single-nucleotide polymorphisms (SNPs) or haplotypes in the TNF/LTA region in an Australian sample by analysing 26 SNPs in 958 endometriosis cases and 959 unrelated controls. We selected functional SNPs in the coding and the promoter region of the TNF gene and HapMap tagging SNPs and typed them on a Sequenom MassARRAY platform. A key SNP (rs1800630) in the promoter region typed in previous studies did not give reliable results. Therefore, we also examined a statistically identical ($r^2 = 1$) SNP (siSNP) (rs2844482), identified using the web based program ssSNPer.

RESULTS: Genotype completion rate was 99.5% for SNPs spanning a region of 15.5 kb across the TNF/LTA locus. There was no evidence for association between endometriosis and TNF/LTA SNPs or SNP haplotypes in our case-control study.

CONCLUSIONS: Our data suggest both TNF and LTA genes are not major susceptibility genes for endometriosis.

Keywords: endometriosis; tumour necrosis factor; single-nucleotide polymorphism; haplotype; lymphotoxin alpha

Introduction

Endometriosis (MIM 131200) is a complex disease characterized by the presence and growth of abnormal endometrial tissue outside the uterus (Giudice and Kao, 2004). The disease occurs in 8–10% of women of reproductive age (Harada et al., 1999; Treloar et al., 1999), but the reasons for establishment and progression of endometriosis remain obscure. A profound inflammatory response has been observed surrounding endometriotic implants, and immune cell infiltration, fibroblast mobilization and connective tissue proliferation may contribute to development of the disease (Dmowski et al., 1981; Oral et al., 1996; Kayisli et al., 2002). Numerous factors regulate the growth or maintenance of endometriotic implants including ovarian steroid hormones and cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF, OMIM 191160) (Fakh et al., 1987; Cummings and Metcalf, 1995; Wu and Ho, 2003; Rae et al., 2004). The elevated concentrations of various cytokines in the peritoneal fluid and peripheral blood of patients with endometriosis suggest aberrant immunologic mechanisms contribute to endometriosis susceptibility (Eisermann et al., 1989; Richter et al., 1998; Iwabe et al., 2002; Durai et al., 2003; Agic et al., 2006). TNF and lymphotoxin alpha (LTA, TNF-beta, OMIM153440) belong to the TNF superfamily and are located next to each other within the major histocompatibility complex class III region on chromosome 6p21.3 (Misawa et al., 2000; Cross et al., 2005). TNF is a pro-inflammatory cytokine with a wide range of immunoregulatory effects and plays a critical role in a number of infectious, inflammatory and autoimmune diseases, including diabetes, asthma, arthritis and possibly endometriosis (Criswell et al., 2004; Randolph et al., 2005; Richter et al., 2005; Zeggini et al., 2005). Secretion of TNF from a variety of cell types, including fibroblasts, immune cells, vascular cells and epithelial cells in women with endometriosis may contribute to the development of this disease by promoting endometrial epithelial cell adherence and proliferation outside the uterine cavity (Hunt et al., 1992; Philippeaux and Piguet, 1993; Tabibzadeh et al., 1995; Chegini et al., 1999; Blomgren et al., 2001; Szyllo et al., 2003; Debrock et al., 2006). Increased concentrations of TNF have been reported...
in endometriosis patients and positively correlated with the degree of disease (Richter et al., 1998). TNF increases macrophage migration inhibitory factor (MIF) gene expression and protein synthesis in human endometrial stromal cells (Cao et al., 2006) and MIF released by ectopic endometrial cells is known as a potent mitogenic factor for human endothelial cells (Yang et al., 2000). TNF may also contribute to angiogenesis in endometriotic lesions (Maas et al., 2001). Elevated concentrations of TNF were found in the peritoneal fluid and granulosa cells of women with endometriosis (Rana et al., 1996; Richter et al., 1998; Barcz et al., 2000; Bedaiwy et al., 2002; Braun et al., 2002; Bullimore, 2003; Durai et al., 2003; Galo et al., 2005) suggesting that TNF may play an important role in endometriosis associated immunoinflammatory changes (Dmowski et al., 1989; Sakamoto et al., 2003). Further evidence for a role for TNF in endometriosis comes from studies in baboons, where treatment with anti-TNF antibody reduced the extent of experimentally induced disease (Falconer et al., 2006). Together, these data support a role for TNF in the pathogenesis of endometriosis.

It is unclear whether the inflammatory responses and altered expression of TNF are a cause or a consequence of endometriotic lesions. One approach to address the direction of causation is to analyse genetic association between endometriosis and variation in TNF. Endometriosis is a complex disease and there is extensive evidence that genes influence disease susceptibility (Kennedy, 1998; Zondervan et al., 2001; Simpson and Bischoff, 2002; Stefansson et al., 2002; Vigano et al., 2003). Several polymorphisms have been described within the promoter region of TNF (Hsieh et al., 2002; Wieser et al., 2002; Darai et al., 2003; Asghar et al., 2004; Teramoto et al., 2004). Variation in the promoter region increases transcriptional activity and production of TNF (Wilson et al., 1997; Kaluza et al., 2000) and is associated with various inflammatory and immune-mediated diseases including meningococcal disease, insulin resistance syndrome, rheumatoid arthritis and Crohn’s disease (Verjans et al., 1994; Nadel et al., 1996; Negoro et al., 1999; Rasmussen et al., 2000). Variation in TNF has been associated with endometriosis in some, but not all studies. In Japanese patients, the -1031C (rs1799964) allele was associated with decreased risk of endometriosis (Asghar et al., 2004) and a haplotype defined by three single-nucleotide polymorphisms (SNPs) in the TNF promoter (-1031T/C, -863C/A and -857C/T; rs1799964, rs1800630 and rs1799724, respectively) was significantly associated with endometriosis (Teramoto et al., 2004). In contrast, there was no association between TNF variants and endometriosis in studies in Korean or Austrian populations (Lee et al., 2002; Wieser et al., 2002). The same SNPs were not typed in all studies and differences in the results may relate to choice of markers, linkage disequilibrium (LD) in the region, study power or population differences.

Most studies on association between expression and disease have focused specifically on TNF. However, the TNF gene is located in a cluster of cytokines including LTA which may share some regulatory elements with TNF (Knight et al., 2003). Both genes have similar biological activities and share common receptors (Hehlangs and Pfeffer, 2005). TNF and LTA both function as homo-trimers and mediate their biological effects through interactions with TNF receptor 1 and TNF receptor 2 on a range of leukocytes and parenchymal cells (Hehlangs and Pfeffer, 2005). Variation in the promoter region of TNF associated with endometriosis could mediate effects either through TNF or LTA. This possibility was demonstrated in a recent study that showed the SNP located at nucleotide -308 in the promoter of the TNF gene, previously thought to regulate TNF gene transcription, was instead involved in RNA polymerase II binding and allele-specific LTA gene transcription (Knight et al., 2003).

Since endometriosis is associated with altered concentrations of TNF and with variation in the promoter region of TNF in some studies, we hypothesized that variation in the TNF/LTA gene complex may be associated with susceptibility to endometriosis in our Australian sample of endometriosis patients. We typed functional variants in the promoter region supplemented with non-redundant tagging SNPs across the TNF/LTA region selected from HapMap in a large Australian case–control sample.

Materials and Methods

Samples

Nine hundred and fifty-eight cases with endometriosis were drawn from our Australian study of endometriosis (Trelor et al., 2002). The vast majority of cases (n = 926) were drawn from families with affected sister pairs (ASPs) (one per family), and 32 were from non-ASP families. All women classified as affected had surgically confirmed endometriosis (Trelor et al., 2002, 2005). Disease severity was assessed retrospectively from medical records using a modified version of Revised American Fertility Society (rAFS) criteria (American Fertility Society, 1985) (Trelor et al., 2005). Cases in our study were recruited from the general population and we obtained confirmation of diagnosis from clinicians and hospitals where the participant had been surgically diagnosed or treated in the past (Trelor et al., 2002, 2005). Almost all our endometriosis cases were assigned a retrospective rAFS stage of disease from I to IV by either a diagnosing/treating clinician or by our clinical consultant (Dr Daniel O’Connor) on the basis of surgical notes in medical records (Trelor et al., 2002). However, the time from diagnosis to recruitment was variable and there was variable detail in the clinical records available. We therefore collapsed stage of disease into two classes for analysis (rAFS I/II and rAFS III/IV) (Trelor et al., 2005). Fifty nine percentage of cases were classified with minimal to mild endometriosis (rAFS stages I/II). The remaining 41% of cases with moderate to severe (rAFS stages III/IV) endometriosis were more likely to have ovarian endometriosis.

A similar number of 959 unrelated controls were drawn from women who volunteered for a twin study of gynecological health (Trelor et al., 1999). Controls were considered at low risk for endometriosis, based on self-reporting that they had never been diagnosed with endometriosis and information from medical records where available. Twins had been asked simply ‘have you had endometriosis?’ (Trelor et al., 1999). They were also asked whether they had ever had a laparoscopy and/or a hysterectomy and the reasons for each. About 27% of control women reported having a hysterectomy and/or laparoscopy. No evidence of endometriosis was reported at any of these procedures (Zhao et al., 2006). The mean ages (±SD) of the cases and controls at the time of data collection were 35.82 ± 8.87 years (range: 17–65) and 45.60 ± 11.98 years.
(range: 29–90), respectively. Ethics approval was obtained from the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Australian Twin Registry. Genomic DNAs were extracted (Miller et al., 1988), and diluted to a working concentration of 2.5 ng/μl. The case and control DNAs were randomly placed in 384-well PCR plates.

**SNP selection**

We selected functional and tagging SNPs across the TNF region based on data from publications and public databases including the International HapMap Project (http://www.hapmap.org/), and The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Five additional functional SNPs in the TNF promoter region reported in previous studies and seven SNPs at putative transcription factor binding sites from the PupaSNP database (http://pupasnp.bioinfo.ochiai.fib.es/) were also selected for this study (Table 1). A total of 34 SNPs were selected and spanned a region of 15.5 kb including the TNF and LTA genes. All SNP sequences were downloaded from the Chip Bioinformatics database (http://snpper.chip.org/) and the sequences were cross-checked in NCBI before assay design.

**Genotyping**

Multiplex assays were designed for 34 SNPs across the TNF/LTA gene locus using the Sequenom MassARRAY Assay Design software (version 3.0). SNPs were typed using iPLEX™ chemistry on a matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Sequenom Inc., San Diego, USA). The 2.5 μl PCR reactions were performed in standard 384-well plates using 10 ng genomic DNA, 0.5 unit of Taq polymerase (HotStarTaq, Qiagen, Valencia, CA, USA), 500 μmol of each dNTP, and 100 nmol of each PCR primer. Standard PCR thermal cycling conditions and post-PCR extension reactions were carried out as described previously (Zhao et al., 2006). The iPLEX reaction products were desalted by diluting samples with 15 μl of water and adding 3 μl of resin. The products were spotted on a SpectroChip (Sequenom Inc.), and data were processed and analysed in a compact mass spectrometer by MassARRAY Workstation (version 3.4) software (Sequenom).

**Statistical analysis**

The TNF/LTA genotypes were inspected and results were tested for departures from Hardy–Weinberg equilibrium (HWE) separately for departure from Hardy–Weinberg equilibrium (HWE) separately for cases and controls using the PEDSTATS program (http://www.sph.umich.edu/csg/abecasis/PedStats/index.html).

Standard statistical programs Haplovew version 3.32 (Whitehead Institute for Biomedical Research, USA) and UNPHASED/COCAPHASE were used to test association between endometriosis and individual SNPs or SNP haplotypes (Dudbridge 2003). Global P-values were obtained for each marker or each haplotype by performing 10,000 permutation tests. Haplotype frequencies and LD estimates were determined by Haplovew (Barrett et al., 2005) using the default method of Gabriel (Gabriel et al., 2002). A global P-value <0.05 was considered to be statistically significant.

**Results**

We typed 34 SNPs spanning a region of 15.5 kb across the TNF/LTA locus in 958 endometriosis cases and 959 unrelated controls. Genotype completion rate was 99.5%. Of the 34 markers, seven were not polymorphic (monomorphic) and were not analysed further.

Genotypes for rs1800630 (-863C/A) showed significant departures from HWE (Fig. 1a). No obvious genotyping errors were apparent (Fig. 1b). Inspection of the sequence through the PupaSNP database (http://pupasnp.bioinfo.ochiai.fib.es/) found two SNPs located within the extension primer sequence on the reverse strand (Fig. 1c). In addition, design using the forward sequence for SNP rs1800630 failed because of incompatibilities with other SNP primers within the multiplex. Data associated with this marker were excluded from further analysis and in order to investigate this important TNF gene promoter SNP, we selected and typed a statistically identical SNP (siSNP) rs2844482 in perfect LD ($r^2 = 1.0$) with rs1800630 from the HapMap database (Nyholt, 2006).

A total of 26 polymorphic SNPs across the TNF/LTA gene locus were analysed (Fig. 2a) including rs2844482. Figure 2b shows a LD plot of SNPs in the TNF/LTA gene locus and Fig. 2c shows the common haplotypes. The minor allele frequencies of the 26 SNPs in the TNF/LTA gene locus ranged from 0.001 to 0.381 in our control samples and 0.002 to 0.399 in our case samples (Table 2). The minor allele frequencies of the four functional polymorphisms (-238G/A, -863C/A, -857C/T and -1031T/C) located in the TNF gene promoter upstream of the LTA 3'UTR region were 5, 19, 9 and 20% in the endometriosis cases and 6, 21, 7 and 20% in controls, respectively. Nominal differences were observed in allele

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**Table 1:** Five functional SNPs in the TNF promoter region and seven SNPs at putative transcription factor binding sites of the TNF/LTA locus genotyped in endometriosis cases and controls

<table>
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<td></td>
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<tr>
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<td>-308G/A</td>
<td>Wilson et al. (1992)</td>
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<td>-238G/A</td>
<td>D’Alfonso and Richiardi (1994)</td>
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*dbSNP rs, NCBI Database SNP reference sequence.*

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frequencies for SNPs -857C/T (rs1799724) and rs1800610 between endometriosis cases and unrelated controls in our study. However, these differences were not significant after correcting for multiple testing, with permutations producing global $P$-values of 0.12 and 0.14 for markers -857C/T and rs1800610, respectively. We found no evidence for association between endometriosis and individual SNPs in the TNF/LTA gene locus for either the allelic or the genotypic association tests. There was modest LD across the TNF/LTA gene locus (Fig. 2b). A single haplotype block was identified with 12 haplotypes at a frequency $>2\%$ in the both case and control samples. Tests of association with SNP haplotypes and endometriosis indicate none were contributing to disease susceptibility (Global $P$-values $>0.05$) (Fig. 2c).

Differences between TNF/LTA allele frequencies on subsets of endometriosis patients and controls were analysed (Table 3). Stratification of cases according to the stage of disease (394 rAFS Stages III/IV cases and 959 controls) gave a best $P$-value of 0.02 for SNP rs1800750, but the global result was non-significant ($P = 0.22$). Tests of association between the cases who self-reported with pelvic pain or menstrual pain with either single SNPs or combinations of SNPs did not reach global significance. Analysis of variation across the TNF/LTA gene locus restricted to the small number of controls (131 individuals) with previous laparoscopy excluding endometriosis also showed no evidence for association (data not shown).

**Discussion**

Susceptibility to endometriosis in Japanese populations is associated with variation in the TNF gene promoter (Asghar et al., 2004; Teramoto et al., 2004). We genotyped 26 functional and HapMap tagging SNPs across the TNF/LTA gene locus and found no evidence for association in a large sample of Australian cases and controls.

In the Japanese studies, Asghar et al. (2004) found the TNF gene promoter SNP -1031T/C (rs1799964) was associated with stage IV endometriosis comprising 130 cases and 185 controls, whereas Teramoto et al. (2004) found a haplotype defined...
Figure 2: Variation in the human tumour necrosis factor/lymphotoxin alpha (TNF/LTA) region
(a) The genomic structure of the TNF/LTA region showing the location of the 26 SNPs genotyped (numbered) and SNPs typed that were not polymorphic in our sample (grey arrows), (b) linkage disequilibrium plot of SNP estimated as $r^2$ using Haploview and (c) common haplotypes and association analysis with endometriosis
by SNPs -1031T/C, -863C/A and -857C/T (rs1799964, rs1800630 and rs1799724) was associated with endometriosis susceptibility in 123 endometriosis cases and 165 healthy controls. Two other studies failed to show evidence for association between TNF gene promoter SNPs -238G/A (rs1799964) and the two SNPs close to the TNF coding region in small samples. We genotyped four of the five TNF promoter SNPs and rs2844482 in strong LD with -863C (rs1800630) and -308G/A (rs1800629) and endometriosis susceptibility in Austrian and Korean populations (Lee et al., 2002; Wieser et al., 2002). Differences in results between studies may relate to choice of markers and LD in the region, since different promoter SNPs were genotyped in the Japanese, Austrian and Korean populations. In our samples, there was modest LD (maximum $r^2 = 0.26$) between SNP -1031T/C (rs1799964) and the two promoter SNPs -238G/A (rs1800630) and -308G/A (rs1800629) suggesting association between endometriosis and the distal promoter SNP -1031T/C (rs1799964) may not be detected with SNPs close to the TNF coding region in small samples. We genotyped four of the five TNF promoter SNPs and rs2844482 in strong LD with -863C/A (rs1800630). We found no evidence for association with either distal or proximal promoter variation in our Australian samples. Our control group was drawn from women with self-reports of no previous diagnosis of endometriosis. It is possible that some asymptomatic cases may be present in the control group, but this is unlikely to affect the conclusion from this study for a disease with a prevalence of 8–10% (Moskvina et al., 2005). Other explanations for variable results between studies include study power and ethnic differences.

A number of nuclear transcription factor-binding sites have been identified within the TNF/LTA gene locus that regulate gene transcription and ultimately determine the amount of TNF or LTA produced (Knight et al., 2004; Liu, 2005). Previous association studies of TNF and LTA genes observed that their haplotypes are associated with a high TNF production phenotype, suggesting these polymorphisms should be analysed as haplotypes rather than as individual genotypes (Moffatt and Cookson, 1997; Davies et al., 2000; Lanas et al., 2001). We found modest LD across the TNF/LTA locus with one single haplotype block and no evidence of association with haplotypes across the LTA-TNF region. Our sample has good power to detect gene associations of small to moderate effect as described previously (Zhao et al., 2006). In addition, our cases are highly selected in terms of family history, and compared with a standard case–control association study, this should further increase power to detect gene associations. We did not find any evidence for association between TNF SNPs and endometriosis in the Australian samples of European descent, suggesting that if the risk of endometriosis is influenced by common promoter variation in TNF in this population, the effect size is small and large samples consisting of thousands of cases and controls will be required to confirm and characterise their involvement.

Endometriosis is associated with chronic inflammation. Controlling cytokine synthesis and release is critical for preventing unrestrained inflammation and TNF has been reported to play an essential role in the cytotoxic activity of cell-mediated immunity via TNF receptors or transcription factors in the signalling pathway (Lachapelle et al., 1993; Sakamoto et al., 2003; Hehlgens and Pfeffer, 2005). The overproduction of TNF cytokine is associated with the pathophysiology of numerous diseases including endometriosis (D’Hooghe et al., 2006). Recent studies on the use of anti-TNF drugs suggest...
Table 3: Association analysis of SNPs in phenotype subgroups of endometriosis patients compared with 959 controls

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MAF, minor allele frequency; rAFS, revised American Fertility Society.

References


an anti-TNF monoclonal antibody (mAb, c5N) and recombinant human TNFRSF1A (r-hTBPI) can inhibit development of endometriosis in an established model of endometriosis in female baboons (D’Hooghe et al., 2006; Falconer et al., 2006). TNF is known as a potent mediator and angiogenic cytokine that promotes the production of other cytokines in various cells. Our study suggests that up-regulation of TNF in endometriosis may be a secondary effect and not related to association with functional variants in the gene. Medication blocking the activation of TNF may still be effective in altering disease progression (D’Hooghe et al., 2001; Falconer et al., 2006), although there was no improvement in one endometriosis patient after 6 years of constant use of a TNF blocker medication (Shakiba and Falcone, 2006).

In this study, we examined the association between endometriosis and individual common SNPs and haplotypes in the TNF/LTA gene locus in an Australian population including functional SNPs in the TNF promoter region. Our data does not provide evidence supporting an association between variation in the TNF/LTA locus and endometriosis susceptibility. We conclude that common variants in the TNF/LTA gene do not play a key role in the pathogenesis of endometriosis.

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