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碩士學位請求論文

Polymorphisms of the *TNFA*, *TNFB* and *TNFR*s in Behcet's Disease

베체트병에서의

TNFA, TNFB와 TNFRs의 유전자 다형성

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論 文 概 要

종양괴사인자 (Tumor necrosis factor)는 주요한 pro-inflammatory 사이토카인으로 대식세포 활성화, 세포자살과 같은 다양한 면역기능을 갖고 있어 만성 염증성 질환인 베체트병을 비롯한 여러 염증 질환에서 연구가 되고 있다. 종양괴사인자 발현은 염증반응에서 증가되고, 종양괴사인자 수용체는 종양괴사인자와 결합하여 염증성 유전자의 활성과 특정 유전자를 억제한다. 베체트 환자에서는 종양괴사인자와 수용성의 종양괴사인자 수용체의 농도가 증가되어 있다. 최근 항-종양괴사인자 항체가 베체트병 치료제로 사용되고 있어, 베체트병 환자의 종양괴사인자와 종양괴사인자 수용체 유전자의 발현 조절 부위의 염기 변이를 게놈 DNA에서 분석하게 되었다. *TNFA* 변이형 대립인자 $-1031^{*}C$ (P = 0.026, OR = 1,4), $-863^{*}A$ (P = 0.006, OR = 1.5), 야생형 대립인자 $-308^{*}G$ (P = 0.014, OR = 1.8)와 그들의 동질접합체는 베체트환자에서 건강인에 비해 높은 비율로 나타났다. 또한 이러한 대립인자는 베체트환자의 주 증상인 구강궤양, 피부병변, 외음부궤양, 안병변과 관절염에서 역시 높은 빈도로 나타났다. *TNFA −376G>A* 변이형은 환자와 건강인에서 모두 발견되지 않았고 *TNFA −857C>T*, *−238G>A*, *TNFB +252A>G* 그리고 *TNFRSF1A −383A>C* 빈도는 베체트병 환자군과 건강인 사이에서 통계적으로 유의한 차이가 없었다. 그러나 *TNFRSF1B 196R/R* 은 베체트환자에서 건강인에 비해 0.3배정도 낮은 빈도로 나타났다. 그러나 이는 대립인자 빈도나 증상별로 나누었을 때는 통계적으로 유의한 수치가 나타나지 않았다. PHASE program으로 추정된 *TNFA* $-1031^{*}C-863^{*}A-857^{*}C-308^{*}G-238^{*}G$ 일배체형은 베체트환자에서 건강인에 비해 1.6배 정도의 높은 비율로 나타났고 *TNFA* $-1031^{*}T-863^{*}C-857^{*}C-308^{*}A-238^{*}G$ 일배체형은 0.6배의 비율로 나타났다. 이러한 일배체형들은 대립인자와 마찬가지로

구강궤양, 피부병면, 외음부궤양, 안병변과 관절염을 동반한 베체트환자에서 역시 비슷한 비율로 나타났다. 즉 *TNFA −1031*C, −863*A, −308*G* 대립인자를 포함한 일배체형은 베체트병 환자군에서 정상인보다 유의하게 높았으며, 반대로 이러한 대립인자를 포함하지 않은 일배체형은 정상인에 비해 낮게 나왔다. 결과적으로 *TNFA −1031*C, −863*A, −308*G* 대립인자는 베체트병의 위험도 증가와 연관이 있고, TNF−α의 발현 증가와 관련이 있는 *TNFA* 프로모터 조절부위의 변이와 전사인자의 결합은 베체트병의 감수성을 높이는데 기여한다고 본다.

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Introduction

Behcet' s Disease (BD; MIM 109650) is an immune-mediated disease with a chronic multisystem inflammatory disorder of unclear etiology characterized by recurrent oral and genital ulcer, skin and uveitis lesion. Other manifestations include arthritis, large vessel involvements (LVI), central nervous system (CNS) and gastrointestinal lesions (GI) (Zierhut et al., 2003).

The exact pathogenesis of BD remains unknown however, genetic, immunologic and environmental factors have been suggested to contribute to the development of the disease (Karasneh et al., 2005). It has been assumed that infectious agents, immune mechanisms and genetic factors such as HLA-B51 are involved in the onset of BD (Kaklamani et al., 1998). A T cell mediated immune response seems to be important in the pathogenesis of BD (Gul et al., 2001). $CD4^{+}$ T lymphocytes seem to be the major cell type in inflammatory infiltrates, and increased concentration of Tumor necrosis factor-alpha (TNF-α; MIM 191160), Interleukin (IL) 6, IL-8 and IL-18 have been described (Mege et al., 1993; Oztas et al., 2005).

Recent studies in Turkish multicase families identified several susceptibility loci to BD through a whole-genome linkage analysis. The strongest susceptibility affected genes for BD lies on the 6p22-24 and 12p12-13 (Karasneh et al., 2005). TNF-α and Tumor necrosis factor-beta (TNF-β; MIM 153440) are located on chromosome 6p21. This location has prompted much speculation

about the role of these genes in the etiology of an inflammatory or autoimmune component (Roxo et al., 2003). TNF acts through the two distinct members of the TNF receptor superfamily, *TNFRSF1A* (TNFR1; MIM 191190) and *TNFRSF1B* (TNFR2; MIM 191191) located on chromosome 12p13 and 1p36, respectively.

TNF is a pro-inflammatory cytokine that is secreted by monocytes (TNF-α) or lymphocytes (TNF-β) (Beutler and Cerami, 1989). TNF is multiple functions in the immune response, activation of macrophages and apoptosis. In the *TNFA* promoter and *TNFB* intron region of these genes, several single nucleotide polymorphisms (SNPs) are present (Figure 1) and some of them appear to influence the transcription level of the gene (Higuchi et al., 1998; Wilson et al., 1997; Lee et al., 1997). The molecular mechanisms, by which possibly promoter SNPs in regulatory regions of *TNFA* have important influence TNF-α expression, the associations of transcription factor with such SNPs have been detected. It seems that such interactions between transcription factor and SNPs provide significant information for understanding the allele-specific modulation of the TNF-α expression. Upstream regulatory response *TNFA* promoter variants which bind various transcription factors, NF-kB p50-p50 homodimer, NF-kB p50-p65 heterodimer and Octamer-binding protein (OCT)-1, have been reported to be associated with autoimmune and infectious disease (Udalova et al., 2000; van Heel et al., 2002; Knight et al., 1999). Increased levels of both TNF-α production and NF-kB nuclear translocation have been shown in BD patients. Too much or too

little TNF-α production might be associated with variable patterns of disease pathogenesis (Goldfeld et al., 1990). The *TNFA −1031T>C*, *−863C>A* and *−857C>T* SNPs and overproduction of TNF-α were reported to be implicated in a variety of inflammatory diseases and autoimmune disease, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Crohn's disease (Negoro et al., 1999).

TNF-α and TNF-β send signals to target cells through two homologous homodimeric receptors: TNFR1 and TNFR2 (Derouich-Guergour et al., 2001). TNFR1 and TNFR2 are membrane glycoproteins present on almost all types of cells except erythrocytes. Cell surface expression of these receptors is controlled by various factors, such as cytokines, protein kinases, phosphatases and so on. They also exist as soluble forms following cleavage from membrane and circulate in serum, urine and other biological fluids under normal and pathological situations. These soluble forms of TNFRs are able to bind TNF-α and to act either as agonists at low concentrations (stabilizing the trimeric structure of TNF-α) or as inhibitors at high concentrations (by competing with cell membrane receptors) of TNF-α activity (Gimenezet al., 2003). The TNFR1 pathway is thought to be dominant and has been implicated in most of the known TNF-α effects, including induction of macrophage activity, up-regulation of adhesion molecules, and NF-kB activation (Osborn et al., 1989) Engagement of TNFR1 by TNF activates several transcription factors that include NF-kB and c-Jun/activator protein 1 (AP-1), leading to

up-regulation of a large number of genes involved in inflammatory and immune responses (Wallach et al., 1999). TNFR1 is expressed in all nucleated cells, particularly those susceptible to the cytotoxic action of TNF, and TNFR2 is expressed predominantly in cell of myeloid origin, particularly stimulated T and B lymphocytes (Bridges et al., 2002). TNFRs are part of the TNF pathway and might be implicated in the pathogenesis of BD. Increased levels of sTNFR2 have been reported in the blood of patients with BD (Zierhut et al., 2003). Most functional domains of the *TNFRSF1B* are encoded by separate exons in the gene. Exon 6 encodes a small portion of the transmembrane region and the position of the proteolytic cleavage site that produces the soluble form of TNFR2 (Beltinger et al., 1996). In addition, the *TNFRSF1B M196R* polymorphism may thus modulate the assembly and conformational change of TNFR2. In fact, the position of codon 196 is known to be an important domain for its dimmer or trimer formation (Morita et al., 2001).

The aim of this study is to analyze the association between Behcet' s Disease and SNPs of the regulatory promoter region of *TNFA* and the first intron of the *TNFB* and their receptors, *TNFRSF1A* and *TNFRSF1B*, in a Korean population.

Materials and Methods

Subjects

Two hundred fifty−nine patients (128 male and 131 female) from 16 to 66 years of age registered at the Behcet' s Disease Specialty Clinic of Severance Hospital at the Yonsei University College of Medicine and the Ajou University School of Medicine and 379 healthy Korean controls were included in this case−control study. The prevalence of clinical feature in patients with BD is presented in Table 1. BD was diagnosed according to the clinical criteria of the International Study Group for Behcet' s Disease or the revised criteria of Behcet' s Disease (Wechsler et al., 1990). Informed consent was obtained from all patients and controls.

Genotyping

Genomic DNA was extracted from peripheral blood by using a QIAamp Blood Mini kit (Quiagen, Valencia, CA, USA). Six SNPs in the promoter region of *TNFA* gene, *TNFB +252G>A*, *TNFRSF1A −383A>C* and *TNFRSF1B 196M>R* (Met196Arg, *676T>G*) were analyzed by using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. For detection of the polymorphism, a 10 ㎕ reaction volume containing 15 ng of DNA was amplified with 10 mM Tris (pH 8.0), 40 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 5 pmol each primer and 0.38 unit Taq polymerase (Bioneer, Korea) on GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, U.S.A). The *−1031 T>C* was

amplified with PCR by using primers 5'-ACA AGG CTG ACC AAG AGA GAA-3' and 5'-GTC CCC ATA CTC GAC TTT CAT-3' and was digested with the *Bbs*I restriction enzyme (Soga et al., 2002). The *-863 C>A* was amplified by using primers 5'-GGC TCT GAG GAA TGG GTT AC-3' and 5'-CTA CAT GGC CCT GTC TTC GTT ACG-3' and was digested with the *Tai*I restriction enzyme (Skoog et al., 1999). The reverse primer contained one mismatched nucleotide sequence. Genotyping for *-857C>T* was conducted by using the forward primer 5'-AAG TCG AGT ATG GGG ACC CCC CGT TAA-3' and the reverse primer 5'-CCC CAG TGT GTG GCC ATA TCT TCT T-3' which made it possible to use the restriction enzyme *Hinc*II (Kato et al., 1999). The *G>A* substitution at position *-376* was analyzed by the *Tsp509*I restriction enzyme by using the following primers 5'-CCC CGT TTT CTC TCC CTC AA-3' and 5'-TGT GGT CTG TTT CCT TCT AA-3' (Moukoko et al., 2003). The polymorphic region containing the *Nco*I restriction site at *-308 G>A* was amplified by using primers 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and 5'-TCC TCC CTG CTC CGA TTC CG-3'. The *G>A* transition polymorphism at position *-238* was examined by PCR using primers 5'-CCC AGA AGA CCC CCC TCG GAA CC-3' and 5'-ACC TTC TGT CTC GGT TTC TTC TCC ATC GC-3' and was digested with the *Hpa*II restriction enzyme (Soga et al., 2002). The polymorphic region containing the *Nco*I restriction site in the first intron of the *TNFB* gene was amplified by using the following primers: forward 5'-CCG TGC TTC GTT TGG ACT A-3' and reverse 5'-AGA GGG GTG GAT GCT TGG GTT C-3' (Lee et

al., 2003). The *A>C* substitution at position *−383* in the promoter region of the *TNFRSF1A* gene was analyzed by the *Bgl*II restriction enzyme by using the following primers 5'−TTA TTG CCC CTT GGT GTT TGG TTG−3' and 5'−TTG TGA CGG AGT GAG AAG GGG AGG−3' (Pitts et al., 1998). The *T>G* transition polymorphism in exon 6 of the *TNFRSF1B* was examined by the PCR−RFLP method, using the following primer: forward 5'−ACT CTC CTA TCC TGC CTG CT−3' and reverse 5'−TTC TGG AGT TGG CTG CGT GT−3'. This substitution at codon 196 results in a nonconservative amino acid substitution (methionine [M] to arginine [R]). The PCR products were then digested with *Nla*III (Lee et al., 2003) (Table 2). The digested PCR products were electrophoresis on an 8% or 5% polyacrylamide gel and were stained with ethidium bromide to visualize the DNA fragments.

Statistical analysis

The Hardy−Weinberg equilibrium and Linkage disequilibrium coefficient Lewontin’s D' (|D'|) were performed with the R program v.2.2.0 (http://cran.r−project.org). The statistical significance of the difference in *TNFA*, *TNFB*, *TNFRSF1A* and *TNFRSF1B* allele frequencies between BD patients and controls were examined by the χ^2 or Fisher’s exact test by using SAS version 8.1 (SAS Institute, Cary, NC). Haplotypes of each individual were inferred by using the PHASE program, v.2.0.1 (Stephens et al., 2001). The level of significance was set at $P < 0.05$, and odds ratios (OR) with 95% CIs were calculated for those comparisons

that had significant P value. The P value, where indicated as corrected P (P_c), has been subjected to the Bonferroni correction: the P value was multiplied by the number of comparisons made (6 for *TNFA* SNPs and 17 for *TNFA* haplotypes).

Results

A total of 259 Korean BD patients and 379 controls were genotyped for *TNFA*, *TNFB, TNFRSF1A* and *TNFRSF1B*. The genotype and allele frequencies of the *TNFA*, *TNFB, TNFRSF1A* and *TNFRSF1B* polymorphisms showed in the BD patients and controls, and that were found to be in Hardy−Weinberg equilibrium in healthy controls and BD patients except for *TNFRSF1B 196 M>R*. The frequencies of *TNFA* $-1031^{*}C/^{*}C$ genotype (P = 0.026, OR = 2.4, 95% CI = 1.08 − 5.44) and $-1031^{*}C$ allele (P = 0.026, OR = 1.4, 95% CI = 1.04 − 1.77) were significantly higher in BD patients than in healthy controls (Table 3). The frequencies of *TNFA* $-863^{*}A/^{*}A$ genotype (P = 0.042, OR = 2.6, 95% CI = 1.00 − 6.65) and $-863^{*}A$ allele (P = 0.006, OR = 1.5, 95% CI = 1.12 − 2.01) were significantly increased in patients. Also the frequencies of *TNFA* $-308^{*}G/^{*}G$ genotype (P = 0.021, OR = 1.7, 95% CI = 1.08 − 2.79) and $-308^{*}G$ allele (P = 0.014, OR = 1.8, 95% CI = 1.11 − 2.76) were also more prevalent in BD than in controls, but P_c values were not significant (Table 3). The *TNFA −376G>A* was not polymorphic in the Korean population and was excluded from further analysis. No association was found among *TNFA −857C>T, −238G>A*, *TNFB +252A>G* and *TNFRSF1A −383A>C* with BD. However, the homozygote frequency of *TNFRSF1B 196R/R* was significantly decreased in BD patients compared to controls (P = 0.036, OR = 0.3, 95% CI = 0.08 − 0.99) (Table 4).

The patients were characterized by clinical features whose major / minor symptoms were oral, genital ulcer, skin, ocular lesion, arthritis and LVI. The CNS and GI are uncommon. The frequencies of _TNFA −1031*C_, _−863*A_ and _−308*G_ alleles and their homozygotes were significantly higher in the BD patients suffering from major symptoms (OR = 1.4 - 2.9) and arthritis (OR = 2.7 - 3.1) than in the controls (Table 5). There was no difference between the presence of _TNFA −238*A_, _TNFB +252*A_ or _TNFRSF1A −383*A_ allele the symptoms of BD except for BD with GI, CNS and LVI, respectively (Table 5 − 6).

The linkage disequilibria showed among _TNFA_ promoter polymorphisms in the Korean population (Figure 2). The estimated haplotype frequencies of the _−1031 T>C_, _−863 C>A_, _−857 C>T_, _−308 G>A_ and _−238 G>A_ are presented Table 7. The overall haplotype distributions were significant difference between BD patients and controls by permutation test (P = 0.05). The _TNFA −1031*C−863*A−857*C−308*G−238*G_ haplotype, which contains the _TNFA −1031*C_, _−863*A_ and _−308*G_ three alleles, was found to be significantly prevalent in BD patients (18.5% _vs._ 12.7%, P = 0.004, OR = 1.6). Conversely, the _TNFA −1031*T−863*C−857*C−308*A −238*G_ was protective against BD (4.9% _vs._ 7.7%, P = 0.045, OR = 0.6), but P_c values were not significant (Table 7).

We demonstrated the clinical features of the _TNFA_ haplotype−positive individuals in terms of the frequency of the haplotype, _TNFA −1031*C−863*A−857*C−308*G−238*G_, which contains the three alleles _−1031*C_, _−863*A_ and _−308*G_. Being

compared with the controls, it was significantly increased among patients (OR = 1.6 – 3.3) who suffered from oral and genital ulcer, skin and ocular lesion, arthritis and CNS but not among those who suffered from the LVI or GI. On the other hand, the haplotype *TNFA −1031**T−863**C−857**C−308**A−238**G* was shown to significantly lower in BD patients than in controls (OR = 0.4 – 0.6) (Table 8). Frequency of *TNFA −1031**C−863**C−857**C−308**G −238**A* haplotype in the patients with GI significantly differed from those in the controls, but the number of patients was too small for a statistical analysis (Table 8).

Discussion

In the present study showed the association of polymorphisms in the *TNFA* and *TNFRSF1B* genes with genetic predispose to BD in Korean. As a result, a significant difference between BD patients and controls was observed in the regulatory response promoter polymorphisms of *TNFA* and exon 6 region of *TNFRSF1B 196R*. TNFA −1031*C, −863*A and −308*G alleles have a strong association with BD but no associations ware observed in the other SNPs among BD patients in Koreans. This is similar findings as BD patients from different ethnic groups. The *−1031 T>C*, designated the *C* allele, which has the strong association with BD in UK Caucasoid patients. Moreover *TNFA* promoter two haplotypes were significantly associated with BD and both contained the −1031*C allele. Conversely, the TNF H1 (−1031*T−863*C−857*C−380*G −308*G−238*G) haplotype, which did not have this allelic polymorphism, was protective against the disease (Ahmad et al., 2003).

We found that the frequencies of the TNFA −863*A allele and its homozygote were significantly higher in BD patients than in healthy controls. Polymorphism at position *−863C>A* in the promoter region has been reported to be associated with reduced TNF−α plasma levels (Skoog et al., 1999). The *TNFA −863C>A* on the relative binding affinities of different forms of the NF−kB complex. It was shown that the p50/p50 homodimeric form of this complex had a significantly decreased affinity to its DNA binding

site for *−863A*. Since the p50/p50 homodimer acts as a transcription repressor on binding to its regulatory site in the promoter region of the *TNFA* gene, decreased binding is thought to result in an inadequate down−regulation of *TNFA* gene expression, and thus in increased TNF−α production (Udalova et al., 2000).

There has been much interest in a group of SNPs located at *−376G>A*, *−308G>A* and *−238G>A* with respect to the TNF transcriptional start site (Knight et al., 1999). The lack of association of the *TNFA −376G>A*, *−308G>A* and *−238G>A* alleles with BD has been reported in Turkish, Caucasian and Korean patients (Ates ea al., 2005; Ahmad et al., 2003; Lee et al., 2003). The *TNFA −308G>A* has been shown to be associated with elevated TNF−α transcription activity (Wilson et al., 1997; Kroeger et al., 1997) The minor allele $-308^{*}A$ of *TNFA*, which influences a consensus sequence for a binding site of the transcription factor AP−2, caused stronger transcriptional activation than common allele $-308^{*}G$ in a human B cell line and serum, thus has been associated with increased TNF−α production in vitro (Wilson et al., 1997; Gonzalez et al., 2003) However in this study, the frequencies of the *TNFA* $-308^{*}G$ allele and its homozygote were significantly higher in BD patients than in controls. It was difficult to proper explanation of this result. But it is known that the production of TNF−α is regulated by *TNFA* promoter response elements and several cytokines such as IL−6, IL−8 and IL−18. In the present study, the *TNFA −376G>A* variant was absent in the Koreans, but was present among Turkish (0.015), Gambian (0.015) and Sudaness

(0.035) (Duymaz-Tozkir et al., 2003; Knight et al., 1999; Moukko et al., 2003). The OCT-1 transcription factor is associated with the SNP site at position *-376*, which appears to affect TNF-α expression: the *-376*A* allele appears to enhance the TNF-α expression as compared with the *-376*G* allele, and to be associated with OCT-1 (Knight et al., 1999).

TNFR1 and TNFR2 are recognized by both TNF-α and TNF-β. The majority of biologic responses classically attributed to TNF are mediated by TNFR1. On the other hand, TNFR2 has been proposed to function as both a TNF antagonist by neutralizing TNF and as a TNF agonist by the interaction between TNF and TNFR1 at the cell surface (Tartaglia et al., 1993). The *TNFRSF1B M196R* has been shown associated with susceptibility and disease course in autoimmune disorders, such as SLE and RA (Morita et al., 2001; Barton et al., 2001). In this study, the distribution of the *TNFRSF1B 196R/R* homozygote was significantly decreased in BD patients compared with controls (P = 0.036, OR = 0.3, 95% CI = 0.08 - 0.99). But no difference was found between Korean BD patients and controls in distributions of *TNFRSF1B M196R* polymorphisms (Lee et al., 2003).

At the *TNFB +252A>G* polymorphism, the presence of *TNFB +252*G* defines the mutant allele known as TNF-β (5.5 kb), whereas *TNFB +252*A* defines the wild-type allele, TNF-β (10.5 kb) (Warzocha et al., 1998). The *TNFB +252*A* homozygote genotype is associated with high TNF (Verity et al., 1999). In this study, the frequencies of the *TNFB +252*A* allele and its

homozygote were higher in BD with CNS patients than in healthy controls, but the number of patients was too small for a statistical analysis. Also TNFB +252*A allele and its homozygote were difference between BD with ocular patients and controls, however not significantly. In Palestinian and Jordanian descendants, the allele frequency of TNFB +252*A was increased in blind BD patients, although this frequency was similar in all BD patients and healthy controls (Verity et al., 1999). In Japanese, the frequency of the TNFB +252*G homozygote was found to be considerably decreased in ocular BD patients (Mizuki et al., 1992).

The *TNFA* polymorphisms were analyzed linkage disequilibrium and the haplotypes. When *TNFA* promoter haplotypes were studied, the TNFA −1031*C−863*A−857*C−308*G−238*G haplotype was significantly associated with BD which contained −1031*C, −863*A and −308*G alleles. Conversely, the TNFA −1031*T−863*C−857*C −308*A−238*G haplotype reduced the susceptibility of BD. Previous studies reported conflicting results on the promoter activity of TNFA −1031*C−863*A−857*C−308*G−238*G increased (Higuchi et al., 1998; Tsuchiya et al., 2001). In fact, TNFA−1031*C−863*A −857*C−308*G−238*G was recently demonstrated to show higher binding to the transcription factor OCT−1, compared with the common TNFA−1031*T−863*C−857*C−308*G−238*G haplotype (Hohjoh and Tokunaga, 2001). The TNFA −857*C allele might have a functional effect on higher stimulated TNF production. The TNF −857*C allele ablates OCT−1 binding at this site, and might therefore act to further augment the NF−kB−mediated inflammatory

response. (van Heel et al., 2002). OCT−1 is transcription factor whose interaction with other proteins leads to diverse effects on gene regulation, in most cases acting to enhance (Kaushansky et al., 1994). Accordingly, differences in the TNF−α expression due to the allele−specific binding of OCT−1 to *TNFA −863C>A*, *−857C>T* and *−376G>A* SNPs maybe contribute to the susceptibilities to various human diseases (Hohjoh and Tokunaga, 2001).

In conclusion, the results showed that the *TNFA* $-1031^{*}C$, $-863^{*}A$ and $-308^{*}G$ alleles were associated with the development of BD and was responsible for the enhanced inflammatory reaction of BD with major symptoms. However, no association was found among *TNFA −857C>T*, *−238G>A*, *TNFB +252A>G* and *TNFRSF1A −383A>C* with BD. These results suggested that the *TNFA* $-1031^{*}C$ and $-863^{*}A$ alleles in *TNFA* promoter response elements may have more transcription effect on the etiopathogenesis of BD.

Table 1. Clinical characteristics of patients with Behcet's Disease

Clinical features	n = 259	(%)
Female / Male	131 / 128	(50.6 / 49.4)
Age (years range)	16 – 66	
Age onset	3 – 58	
Major symptoms		
Oral ulcers	259	(100.0)
Skin lesions	232	(89.6)
Genital ulcers	212	(81.9)
Ocular lesions	177	(68.3)
Minor symptoms		
Arthritis	119	(45.9)
Large vessel involvement	44	(17.0)
Central nervous system involvement	14	(5.4)
Gastrointestinal lesions	14	(5.4)

Table 2. Sequences of amplifying primers for *TNFA*, *TNFB*, *TNFRSF1A* and *TNFRSF1B* SNPs genotyping by PCR-RFLP

Gene	Loci	Primer sequence	Restriction enzyme
TNFA	*-1031 T>C*	F: ACAAGGCTGACCAAGAGAGAA	*Bbs*I: 521bp: 360, 161bp
		R: GTCCCCATACTCGACTTTCAT	Soga et al., 2002
	-863 C>A	F: GGCTCTGAGGAATGGGTTAC	*Tai*I: 125bp: 102, 23bp
		R: CTACATGGCCCTGTCTTCGTTACG	Skoog et al., 1999
	-857 C>T	F: AAGTCGAGTATGGGGACCCCCCGTTAA	*Hinc*II: 133bp: 108, 25bp
		R: CCCCAGTGTGTGGCCATATCTTCTT	Kato et al., 1999
	-376 G>A	F: CCCCGTTTTCTCTCCCTCAA	*Tsp*509I : 106bp: 86, 20bp
		R: TGTGGTCTGTTTCCTTCTAA	Moukoko et al., 2003
	-308 G>A	F: AGGCAATAGGTTTTGAGGGCCAT	*Nco*I : 107bp, 87, 20bp
		R: TCCTCCCTGCTCCGATTCCG	Wilson et al., 1992
	-238 G>A	F: CCCAGAAGACCCCCCTCGGAACC	*Hpa*II : 126bp, 104, 20bp
		R: ACCTTCTGTCTCGGTTTCTTCTCCATCGC	Soga et al., 2002
TNFB	*+252 A>G*	F: CCGTGCTTCGTGCTTTGGACTA	*Nco*I: 740bp: 555, 175bp
		R: AGAGGGGTGGATGCTTGGGTTC	Lee et al., 2003
TNFRSF1A	*-383 A>C*	F: TTATTGCCCCTTGGTGTTTGGTTG	*Bgl*II: 199bp: 135, 64bp
		R: TTGTGACGGAGTGAGAAGGGGAGG	Pitts et al., 1998
TNFRSF1B	*196 M>R*	F : ACTCTCCTATCCTGCCTGCT	*Nla*III: 242bp: 132, 110bp
		R : TTCTGGAGTTGGCTGCGTGT	Lee et al., 2003

Mismatched bases are underlined

Table 3. Genotype and allele frequencies of the *TNFA* SNPs in BD patients and in controls

Gene	Region	Polymorphism		BD n = 259 (%)	Controls n = 379 (%)	*P*	OR (95% CI)
TNFA	Promoter	-1031T>C	-1031*T/*T	146 (56.4)	240 (63.3)		
		(rs1799964)	-1031*T/*C	97 (37.4)	129 (34.0)		
			-1031*C/*C	16 (6.2)	10 (2.7)	0.026	2.4 (1.08- 5.44)
			-1031*C	0.249	0.197	0.026	1.4 (1.04- 1.77)
		-863C>A	-863*C/*C	163 (63.0)	273 (72.0)	0.015	0.7 (0.47- 0.92)
		(rs1800630)	-863*C/*A	84 (32.4)	99 (26.1)		
			-863*A/*A	12 (4.6)	7 (1.9)	0.042	2.6 (1.00- 6.65)
			-863*A	0.208	0.149	0.006	1.5 (1.12- 2.01)
		-857C>T	-857*C/*C	191 (73.8)	270 (71.2)		
		(rs1799724)	-857*C/*T	64 (24.7)	97 (25.6)		
			-857*T/*T	4 (1.5)	12 (3.2)		
			-857*C	0.861	0.840		
		-376G>A	-376*G/*G	259 (100.0)	379 (100.0)		
		(rs1800750)	-376*G/*A	0 (0.0)	0 (0.0)		
			-376*A/*A	0 (0.0)	0 (0.0)		
			-376*A	0	0		
		-308G>A	-308*G/*G	231 (89.2)	313 (82.6)	0.021	1.7 (1.08- 2.79)
		(rs1800629)	-308*G/*A	28 (10.8)	63 (16.6)		
			-308*A/*A	0 (0.0)	3 (0.8)		
			-308*G	0.946	0.909	0.014	1.8 (1.11- 2.76)
		-238G>A	-238*G/*G	238 (91.9)	360 (95.0)		
		(rs361525)	-238*G/*A	21 (8.1)	17 (4.5)		
			-238*A/*A	0 (0.0)	2 (0.5)		
			-238*A	0.041	0.028		

Table 4. Genotype and allele frequencies of the *TNFB, TNFRSF1A* and *TNFRSF1B* SNPs in BD patients and in controls

Gene	Region	Polymorphism		BD n = 259 (%)	Controls n = 379 (%)	*P*	OR (95% CI)
TNFB	intron	+252A>G	+252*A/*A	95 (36.7)	122 (32.2)		
			+252*A/*G	126 (48.6)	185 (48.8)		
			+252*G/*G	38 (14.7)	72 (19.0)		
			+252*A	0.610	0.566		
TNFRSF1A	promoter	-383A>C	-383*A/*A	215 (83.0)	306 (80.7)		
		(rs2234649)	-383*A/*C	44 (17.0)	67 (17.7)		
			-383*C/*C	0 (0.0)	6 (1.6)		
			-383*A	0.915	0.896		
TNFRSF1B	Exon 6	*196M>R*	*196M/M*	164 (63.3)	262 (69.1)		
		(rs17883437)	*196M/R*	92 (35.5)	102 (26.9)		
			196R/R	3 (1.2)	15 (4.0)	0.036	0.3 (0.08- 0.99)
			196R	0.189	0.174		

Table 5. The allele frequencies of *TNFA* for clinical features of BD patients

	n	*-1031*C*	*P*	OR (95% CI)	*-863*A*	*P*	OR (95% CI)	*-308*G*	*P*	OR (95% CI)	*-238*G*	*P*	OR (95% CI)
Controls	379	0.197			0.149			0.909			0.972		
BD	259	0.249	0.026	1.4 (1.04- 1.77)	0.208	0.006	1.5 (1.12- 2.01)	0.946	0.014	1.8 (1.11- 2.76)	0.959		
Oral ulcer	259	0.249	0.026	1.4 (1.04- 1.77)	0.208	0.006	1.5 (1.12- 2.01)	0.946	0.014	1.8 (1.11- 2.76)	0.959		
Skin lesion	232	0.254	0.018	1.4 (1.06- 1.84)	0.211	0.005	1.5 (1.13- 2.06)	0.948	0.012	1.8 (1.14- 2.97)	0.957		
Genital ulcer	212	0.264	0.007	1.5 (1.11- 1.94)	0.219	0.002	1.6 (1.18- 2.18)	0.948	0.016	1.8 (1.12- 3.00)	0.960		
Ocular lesion	177	0.263	0.013	1.5 (1.08- 1.96)	0.218	0.005	1.6 (1.15- 2.19)	0.946	0.032	1.8 (1.05- 2.98)	0.960		
Arthritis	119	0.265	0.025	1.5 (1.05- 2.07)	0.227	0.005	1.7 (1.16- 2.41)	0.966	0.004	2.9 (1.36- 6.08)	0.966		
LVI	44	0.250			0.216			0.898			0.966		
CNS	14	0.429	0.003	3.1 (1.42- 6.62)	0.321	0.014	2.7 (1.19- 6.13)	0.964			0.929		
GI	14	0.321			0.143			0.964			0.893	0.041	0.2 (0.07- 0.85)

LVI : Large vessel involvement, CNS : Central nervous system involvement, GI : Gastrointestinal lesions

P : BD patients *vs.* controls

Table 6. The allele frequencies of *TNFB*, *TNFRSF1A* and *TNFRSF1B* for clinical features of BD patients

	n	*TNFB* $+252^{*}A$	*P*	OR (95% CI)	*TNFRSF1A* $-383^{*}A$	*P*	OR (95% CI)	*TNFRSF1B 196R*	*P*	OR (95% CI)
Controls	379	0.566			0.896			0.174		
BD	259	0.610			0.915			0.189		
Oral ulcer	259	0.610			0.915			0.189		
Skin lesion	232	0.616			0.920			0.179		
Genital ulcer	212	0.623			0.925			0.189		
Ocular lesion	177	0.605			0.907			0.175		
Arthritis	119	0.605			0.916			0.181		
LVI	44	0.580			0.966	0.035	3.3 (1.02-10.67)	0.216		
CNS	14	0.786	0.021	2.8 (1.13- 7.01)	0.929			0.143		
GI	14	0.607			0.857			0.179		

P : BD patients *vs.* controls

Table 7. Haplotype frequencies of *TNFA* in BD patients and in controls

Haplotype	BD (%) n = 518	Controls (%) n = 758	*P*	OR (95% CI)
*-1031*T-863*C-857*C-308*G-238*G*	54.9	55.1		
*-1031*T-863*C-857*T-308*G-238*G*	12.9	14.6		
*-1031*C-863*A-857*C-308*G-238*G*	18.5	12.7	0.004	1.6 (1.15- 2.13)
*-1031*T-863*C-857*C-308*A-238*G*	4.9	7.7	0.045	0.6 (0.38- 0.99)
*-1031*C-863*C-857*C-308*G-238*G*	2.4	3.9		
*-1031*C-863*C-857*C-308*G-238*A*	2.9	2.0		
*-1031*T-863*A-857*C-308*G-238*G*	1.2	1.2		
Others	2.3	2.8		

P : BD patients *vs.* controls

Table 8. Clinical features associated with *TNFA* haplotypes

Haplotype	Controls n = 758	BD n = 518	Oral ulcer n = 518	Skin lesion n = 464	Genital ulcer n = 424	Ocular lesion n = 354	Arthritis n = 238	LVI n = 88	CNS n = 28	GI n = 28
-1031T>C- -863C>A- -857C>T- -308G>A- -238G>A										
T-C-C-G-G	0.551	0.549	0.549	0.550	0.540	0.543	0.551	0.504	0.476	0.468
T-C-T-G-G	0.146	0.129	0.129	0.122	0.124	0.127	0.122	0.109	0.071	0.170
C-A-C-G-G	0.127	0.185	0.185	0.188	0.196	0.202	0.196	0.191	0.321	0.141
P		***P* = 0.004**	***P* = 0.004**	***P* = 0.004**	***P* = 0.002**	***P* = 0.0009**	***P* = 0.007**		***P* = 0.005**	
OR (95% CI)		1.6 (1.15- 2.13)	1.6 (1.15- 2.13)	1.6 (1.16- 2.18)	1.7 (1.22- 2.31)	1.8 (1.26- 2.46)	1.7 (1.16- 2.49)		3.3 (1.44- 7.43)	
T-C-C-A-G	0.077	0.049	0.049	0.048	0.048	0.050	0.031	0.094	0.023	0.028
		***P* = 0.045**	***P* = 0.045**	***P* = 0.046**	***P* = 0.051**		***P* = 0.010**			
		0.6 (0.38- 0.99)	0.6 (0.38- 0.99)	0.6 (0.36- 1.00)	0.6 (0.35- 1.01)		0.4 (0.16- 0.81)			
C-C-C-G-G	0.039	0.024	0.024	0.026	0.029	0.023	0.034	0.043	0.024	0.076
C-C-C-G-A	0.020	0.029	0.029	0.032	0.029	0.032	0.025	0.004	0.070	0.103
										***P* = 0.020**
										5.9 (1.62-21.86)
T-A-C-G-G	0.012	0.012	0.012	0.013	0.011	0.011	0.019	0.010	0.000	0.001
Others	0.028	0.023	0.023	0.021	0.023	0.012	0.022	0.045	0.015	0.013

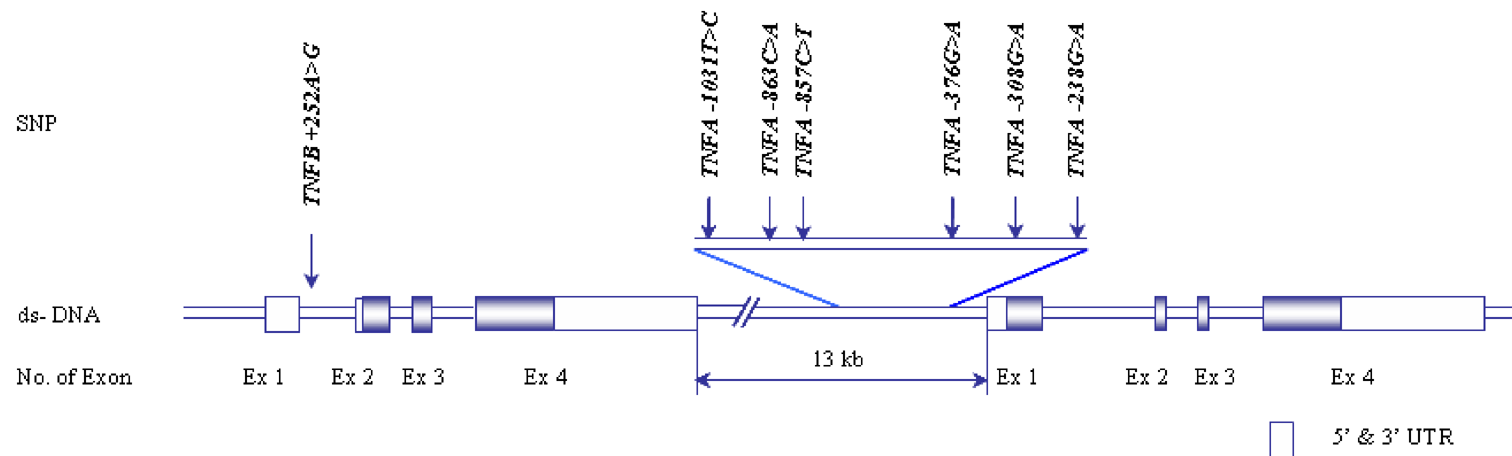

Figure 1. Structural of *TNFA* and *TNFB* genes and location of SNPs

	| D' |				
	-1031T>C	*-863C>A*	*-857C>T*	*-308G>A*	*-238G>A*
-1031T>C	-	0.858	0.998	0.705	0.706
-863C>A	<0.0001	-	0.841	0.571	0.989
-857C>T	<0.0001	<0.0001	-	0.817	0.657
-308G>A	0.002	0.037	0.002	-	0.497
-238G>A	<0.0001	0.054	0.184	0.465	-

P

Figure 2. Linkage disequilibria (|D'|) among SNP of *TNFA* in the Korean population (n=379)

|D'| value is given above the diagonal; *P*-value is given below the diagonal

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ABSTRACT

Polymorphisms of the *TNFA*, *TNFB* and *TNFR*s in Behcet's Disease

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The etiology of Behcet’ s Disease (BD) is uncertain, cytokines play a pathologic role in the development of BD. Tumor necrosis factor (TNF) is an important pro-inflammatory cytokine which has multiple functions in the immune response, activation of macrophages and apoptosis. TNF elevated in patients with active BD, also polymorphisms of *TNFA* and *TNFB* gene have been known to be associated with altered TNF secretion. It seems that such interactions between transcription factor and SNPs provide significant information for understanding the allele-specific modulation of the TNF-α expression. The biological activities of TNF are mediated through the two distinct receptors: the 55-kDa TNF receptor 1 (TNFR1, *TNFRSF1A*) and the 75-kDa TNF receptor 2 (TNFR2, *TNFRSF1B*). TNF receptors are part of the TNF pathway and might be implicated in the pathogenesis of BD. Increased levels of sTNFR2 have been reported in the blood of patients with BD. This study has focused on the association between *TNFA*, *TNFB*, *TNFRSF1A* and *TNFRSF1B* polymorphisms and the corresponding

TNFA haplotypes and BD risk. 259 BD patients and 379 controls were genotyped for the _−1031T>C_, _−863C>A_, _−857C>T_, _−376G>A_, _−308G>A_ and _−238G>A_ polymorphisms of the _TNFA_ gene, _TNFB +252A>G_, _TNFRSF1A −383A>C_ and _TNFRSF1B 196M>R_ by PCR−RFLP method. _TNFA −1031*C_, _−863*A_ and _−308*G_ alleles were associated with an increased risk for BD (_P_ = 0.026, OR = 1.4; _P_ = 0.006, OR = 1.5; _P_ = 0.014, OR = 1.8, respectively). The _TNFA −376G>A_ was not polymorphic in the Korean population and no associations were found among _TNFA −857C>T_, _−238G>A_, _TNFB +252A>G_ and _TNFRSF1A −383A>C_ with BD. Whereas the _TNFRSF1B 196R/R_ was significantly lower in BD patients than in controls (P = 0.036, OR = 0.3). The _TNFA_ haplotype _−1031*C−863*A−857*C−308*G−238*G_ was associated with a 1.6 fold increased risk of BD, whereas the _TNFA_ haplotype _−1031*T−863*C−857*C−308*A−238*G_ with a 0.6 decreased risk of BD. In conclusion, the _TNFA −1031*C_, _−863*A_ and _−308*G_ alleles and their homozygote were found to be associated with BD. These results demonstrate that polymorphisms and the corresponding haplotypes in _TNFA_ promoter response elements may have some transcription effect on the etiopathogenesis of BD.