Concise Report

Influence of VEGF gene polymorphisms on the severity of ankylosing spondylitis

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Objectives. To investigate the role of polymorphisms of the vascular endothelial growth factor (VEGF) gene in susceptibility to ankylosing spondylitis (AS), and their relationship to clinical features and radiographic severity.

Methods. This study included 157 patients with AS and 140 healthy unrelated controls. Polymorphisms of the VEGF gene were analysed by the polymerase chain reaction (PCR)–restriction fragment length polymorphism assay and amplification refractory mutation system–PCR. Haplotypes were reconstructed using the Bayesian algorithm. Radiographic severity was assessed by the Bath Ankylosing Spondylitis Radiological Index (BASRI).

Results. The genotype frequencies of the polymorphisms were in Hardy–Weinberg equilibrium. The distributions of genotypes and alleles did not differ between AS patients and controls. Among the six haplotypes reconstructed based on the tight linkage disequilibrium at positions −2578, −1154 and −634 (pairwise linkage disequilibrium coefficient, \( r = 0.361–0.706 \)), no haplotype was associated with susceptibility to AS. Clinical features were analysed for the four haplotypes (CGC, CGG, AAG, AGG) which were prevalent. In carriers of the AGG haplotype, the frequency of cervical spine involvement was significantly higher (\( P = 0.002, P_{corr} = 0.036 \)) and that of patients showing a BASRI score >6 was also higher (\( P = 0.025, P_{corr} = 0.45 \)).

Conclusions. This study demonstrates that polymorphisms of the VEGF gene may contribute to disease severity in AS.

Key words: Vascular endothelial growth factor, Ankylosing spondylitis, Polymorphism.

Ankylosing spondylitis (AS) is a chronic inflammatory disorder in which genetic factors play a major role in susceptibility. Although HLA-B27 is important, non-major histocompatibility complex (MHC) genes are estimated to account for at least half of the genetic variability for AS [1]. Case-control studies using candidate non-MHC gene approaches revealed weak associations of several genes, including IL-1Ra, CYP2D6 and TGF-β1 [2–5]. As assessed by the Bath Ankylosing Spondylitis Functional Index (BASFI), disease severity has also been shown to be largely genetically determined [6]. However, only a few candidate gene association studies have been performed for disease severity or clinical features in AS, and these showed weak or negative associations [3, 7, 8].

Angiogenesis is an important process in the pathogenesis of chronic inflammatory disorders such as rheumatoid arthritis [9, 10]. The pathological findings of sacroiliitis and peripheral arthritis in AS demonstrated increased vascularity in the synovial tissues [11–13], indicating the importance of angiogenesis in this disease. Among the proangiogenic factors, VEGF plays a central role in human RA and animal models of arthritis [10, 14]. VEGF concentration has been reported to be increased in spondyloarthropathies including AS [15, 16], and showed significant correlations with the disease activity as indicated by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the severity of disease as indicated by the Bath Ankylosing Spondylitis Metrology Index (BASMI).

Single nucleotide polymorphisms (SNPs) of the VEGF gene are associated with production of VEGF protein and are reported to be involved in susceptibility to several disorders in which angiogenesis may be critical in pathogenesis [17–24]. In rheumatoid arthritis, the T allele at position 936 of the VEGF gene is significantly more frequent and the carriers of the two susceptible haplotypes are associated with younger age at disease onset [23].

Considering the similar pathological findings of synovitis in AS compared with those of rheumatoid arthritis, it is possible that polymorphisms of the VEGF gene may have a role in susceptibility to and severity of AS. The purpose of the present study was to assess the potential association between polymorphisms in the VEGF gene and susceptibility to AS. We also examined the effect of the polymorphisms on clinical features, including radiographic severity of AS.

Materials and methods

Study population

One hundred and fifty-seven Korean patients with AS who fulfilled the modified New York criteria [25] were recruited from outpatient clinics of four university hospitals, including Kyungpook National University Hospital, Republic of Korea.
National University Hospital, Chonnam National University Hospital, Pusan National University Hospital and Chonbuk National University Hospital. A control group of 140 unrelated, healthy Korean individuals who had no known medical problems on a health screening questionnaire were enrolled. All individuals gave informed consent for study participation and the study was approved by the Institutional Review Board of each hospital.

Clinical and demographic data, including peripheral arthritis, enthesopathies, uveitis, HLA-B27 and age at disease onset, were collected from AS patients. We determined the severity of AS using the Bath Ankylosing Spondylitis Radiological Index (BASRI) [26] in 147 patients whose X-ray films of sacroiliac joints, lumbar spine and cervical spine were available. The BASRI hip score was not included in this study because of the low frequency of hip involvement.

AS patients included 132 men and 25 women between 17 and 69 yr of age (mean 29.8 yr). The mean duration of the disease and the age at onset were 6.25 and 23.1 yr respectively. Among AS patients, 147 (93.6%) had HLA-B27, 102 (65.2%) peripheral arthritis, 73 (46.5%) enthesitis and 28 (17.8%) uveitis. The control group included 101 men and 39 women between 19 and 52 yr of age (mean 28.8 yr).

Genotyping of the VEGF gene polymorphisms

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) by a standard extraction method. The biallelic Equilibrium transition polymorphisms at position –2578 and –1154 in the 5'-untranslated region (UTR) and position 936 in the 3'-UTR were genotyped according to a previously described method [23].

Statistical analysis

The differences in genotype distribution and allele frequency among the groups were examined for statistical significance using the χ² test and Fisher’s exact test. Allele and genotype frequencies were tested for Hardy–Weinberg equilibrium using the χ² test. For the comparison of mean values, Student’s t-test and analysis of variance were performed. To calculate linkage disequilibrium (LD) coefficients between polymorphisms of the VEGF gene, we used SAS Genetics software (SAS Institute, Cary, NC, USA). Statistical reconstruction of haplotypes in this case-control study was performed with the Bayesian algorithm using the Phase program [27], which is available at http://www.stat.washington.edu/stephens/phase.html. The odds ratios and 95% confidence intervals (CIs) were obtained by unconditional logistic regression analysis. When multiple comparisons were involved, corrected P values were calculated for multiple testing using Bonferroni’s method. All analyses were conducted using SPSS, v. 10.0 (SPSS, Chicago, IL, USA).

Results

The genotype frequencies of the four polymorphisms of the VEGF gene showed no significant deviation from Hardy–Weinberg expectation. No significant differences in genotype and allele frequencies were observed between the AS patients and controls (data not shown). We compared the clinical and demographic features of the AS patients according to the genotype of respective polymorphism of the VEGF gene, and there were no significant differences (data not shown). The frequency of HLA-B27 was totally independent of the genotypes of the four VEGF polymorphisms.

Because linkage disequilibrium has been suggested to be highly structured into conserved blocks of sequence separated by hotspots of recombination, the final function of a conserved haplotype may be the result of interaction among polymorphisms within the block [28, 29]. We calculated the LD coefficient between polymorphisms which showed that –2578, –1154 and –634 polymorphisms are in tight linkage disequilibrium (pairwise LD coefficient r = 0.361–0.706), and that 936 polymorphism was only weakly associated with the other polymorphisms (r = 0.032–0.141). Based upon the LD coefficients, haplotype inference was restricted to three polymorphisms at positions –2578, –1154 and –634. Of the eight possible haplotypes, six were estimated to be present and four (CGC, CGG, AAG and AGG) accounted for more than 98% of patients and controls (Table 1). Frequencies of the inferred haplotypes were not significantly different between the AS group and controls.

We then compared clinical features according to the carriers of each haplotype (Table 2). Of the six haplotypes, four (CGC, CGG, AAG and AGG), which were dominant in both groups, were used for the analysis. The frequency of cervical spinal involvement was significantly higher in the carriers of AGG (P = 0.002, Pcorr = 0.036), who also showed a higher frequency of BASRI score >6 (P = 0.025, Pcorr = 0.45). The BASRI score divided by disease duration was slightly higher in the AGG carriers, but the difference was not statistically significant. Clinical manifestations did not differ according to the presence of CGG, CGC or AAG haplotypes.

Discussion

The present data demonstrate that no associations exist between the four functional polymorphisms of the VEGF gene and susceptibility to AS. Although haplotypes at positions –2578, –1154 and –634 were not associated with AS, carriers of the AGG

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>AS (%)</th>
<th>Controls (%)</th>
<th>P-value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGC</td>
<td>139 (44.3)</td>
<td>124 (44.3)</td>
<td>1.000</td>
<td>0.999 (0.722–1.382)</td>
</tr>
<tr>
<td>CGG</td>
<td>94 (29.9)</td>
<td>90 (32.1)</td>
<td>0.594</td>
<td>0.902 (0.637–1.278)</td>
</tr>
<tr>
<td>AAG</td>
<td>46 (14.6)</td>
<td>36 (12.9)</td>
<td>0.553</td>
<td>1.637 (1.728–1.860)</td>
</tr>
<tr>
<td>AGG</td>
<td>30 (9.6)</td>
<td>29 (10.4)</td>
<td>0.784</td>
<td>0.914 (0.534–1.560)</td>
</tr>
</tbody>
</table>

| TABLE 2. Demographic and clinical findings according to the presence of AGG haplotype of the VEGF gene in patients with AS |
|------------------|------------------|------------------|------------------|------------------|
| Age at onset (yr) | 23.39 ± 0.80     | 21.79 ± 1.36     | 0.382            |
| Males:females     | 108:21           | 24:4             | 1.000            |
| Duration of disease (yr) | 6.12 ± 0.47 | 6.83 ± 1.00 | 0.522            |
| C-spine involvement (%) | 44/120 (36.7) | 19/27 (70.4) | 0.002b           |
| L-spine involvement (%) | 88/120 (73.3) | 22/27 (81.5) | 0.467            |
| BASRI score >6 (%) | 37/120 (30.8) | 15/27 (55.6) | 0.025c           |
| BASRI score/yrn² | 1.66 ± 0.13     | 1.76 ± 0.28     | 0.734            |
| Peripheral arthritis (%) | 92 (63.6) | 20 (71.4) | 0.515            |
| Enthesitis (%)     | 57 (44.2)        | 16 (57.1)       | 0.296            |
| Uveitis (%)        | 24 (18.6)        | 4 (14.3)        | 0.787            |
| Renal involvement (%) | 33 (25.6) | 5 (17.9) | 0.472            |

aValues are mean ± S.E.  
bPcorr = 0.036, Bonferroni correction.  
cPcorr = 0.45, Bonferroni correction.
which is located at the VEGF gene, located on chromosome 6p12, were not associated with disease severity in monozygotic compared with dizygotic twins [36]. Previous results indicate that genes outside the HLA region must be involved in the pathogenesis of sacroiliitis. Synovial membranes show inflammatory processes similar to those of rheumatoid arthritis [12, 13, 30]. In the peripheral arthritis of AS, the degree of increased vascularity is similar to that in RA. AS is characterized by new bone formation that results in anklyoses of intervertebral and sacroiliac joints. Angiogenesis also plays an important role in bone formation from cartilage, during which new vessels grow into cartilage before osteoblastic activation [31]. These findings suggest that angiogenesis is an essential component in the pathogenesis of AS. VEGF gene polymorphisms and haplotypes, which may have interactions between polymorphic sites, have been reported to differentially regulate the production of VEGF [18, 19, 21, 32–35]. A previous report showed that VEGF polymorphism at position 936 and haplotypes at positions −2578, −1154, −634 and 936 are associated with susceptibility to rheumatoid arthritis [23]. In the present study, we could not find any associations of the four functional polymorphisms or the statistically reconstructed haplotypes with susceptibility to AS. The susceptibility to AS has been estimated to be largely determined by genetic factors in monozygotic compared with dizygotic twins [36].

Based on the observation of greater similarity of disease severity in monozygotic compared with dizygotic twins [36], Hamersma et al. [6] demonstrated that in AS disease severity is largely genetically determined and that shared environmental factors play little role in determining the disease severity. Several candidate gene association studies have been performed to assess disease severity and clinical features in AS. Studies of HLA-B27 and other MHC genes, such as HLA-DR and LMP2, showed no association with disease severity indices [41–44], supporting the idea that the genes determining the severity of AS may be encoded outside of the MHC region. IL-10 polymorphism was reported to be weakly associated with BASFI [8], and vitamin D receptor polymorphism showed weak associations with inflammatory indices, such as C-reactive protein and ESR [45]. When AS patients were divided into two groups according to the presence of the AGG haplotype, carriers of the AGG haplotype had a significantly higher frequency of cervical vertebral involvement. The mechanisms that underlie the more extensive spinal involvement in carriers of the AGG haplotype are unknown. Because the VEGF concentration in AS has been reported to be elevated in axial spondyloarthropathy compared with controls [15] and there is a significant correlation with disease severity, indicated by BASMI [16], it is possible that the AGG haplotype is associated with higher production of VEGF in the inflammatory milieu of AS, resulting in more extensive radiographic change.

Of the four selected SNPs on the VEGF gene reported to be associated with VEGF synthesis, the −634 polymorphism, which is located at the 5′-UTR, revealed controversial results. Watson et al. [32] showed that there was higher VEGF production from lipopolysaccharide (LPS) stimulated PBMC in GG homozygotes than in AA homozygotes, and suggested that LPS may work through the myeloid zinc finger protein (MZF1) binding site, within which the +405 (same as −634) polymorphism was predicted to be located. However, the serum concentration was higher in the CC homozygote in healthy individuals [21]. The discrepancy between these results may be partially explained by another study [33]. Among carriers of the −460C+/+450G haplotype, VEGF production was different according to the presence of another polymorphism in between, such as −116G/A. When we further analysed the present data, carriers of the −1154G/−634G haplotype showed a higher frequency of cervical spine involvement (31.4 vs 49.0%, \( P = 0.04 \)), and additional carriage of −2578A was associated with more conspicuous differentiation in radiological severity (36.7 vs 70.4%, \( P = 0.002 \)). Because the VEGF gene is very highly polymorphic, there is the possibility that if the impact of a single polymorphism is not overwhelming, the net effect of multiple polymorphisms may determine the production of VEGF, thus affecting the clinical manifestations. We are currently evaluating the influence of haplotypes of the VEGF gene polymorphisms on the production of VEGF.

Our study indicates that non-MHC region genes may influence the severity of AS. Further studies of the associations between severity indices, such as BASRI, BASFI and BASMI, and genes involved in the inflammatory processes of AS are necessary to clarify how genetic factors determine the severity of the disease.

**Key messages**

- These data support the possibility that non-MHC genes influence the severity of AS.
- Carriers of AGG haplotype at positions −2578, −1154 and −634 of the VEGF gene had more extensive radiological change.

**Acknowledgement**

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The authors have declared no conflicts of interest.

**References**