No Association of Polymorphisms in the Tumor Necrosis Factor Receptor I and Receptor II Genes with Disease Severity in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** A recent Italian study found that homozygosity for the G allele of the +196 single nucleotide polymorphism (SNP) of the tumor necrosis factor receptor II (TNFRSF1B) gene was more prevalent in patients with severe rheumatoid arthritis (RA). We investigated whether this particular SNP, and also one at position +36 in exon 1 of the TNF receptor I (TNFRSF1A) gene, are associated with disease severity.

Methods. A group of 181 Caucasian patients with RA was studied. DNAwas isolated from patient blood samples and subsequently used to genotype both the exon 1 TNFRSF1ASNPand the exon 6 TNFRSF1B SNP by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. Radiographic damage was measured by the Larsen score, and functional outcome was assessed by the Health Assessment Questionnaire (HAQ). Data were analyzed by multiple regression analysis, with correction for age, sex, and disease duration.

Results. The mean Larsen and HAQ scores did not differ significantly between each of the genotypes from the 2 TNFR SNP. No significant associations between the +36 TNFRSF1A SNP or the +196 TNFRSF1B SNP genotypes and disease severity were found after correcting for age, sex, and disease duration.

Conclusion. Our data suggest that neither the +36 TNFRSF1ASNP nor the +196 TNFRSF1B SNP is associated with RA severity in a population of Caucasian patients with RA. (J Rheumatol 2003;30:1406–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS TUMOR NECROSIS FACTOR-α TNFR POLYMORPHISM

Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation of the joints, although the etiology of the disease is currently unknown. The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is a major factor in the generation and maintenance of the inflammatory state observed in RA[1]. The role of TNF-α in the pathology of RA has thus become a significant avenue of research in recent years and has led to the development of new therapies targeted at this cytokine[2-5].

The effects of TNF-α are mediated upon binding to either of 2 distinct cell surface receptor molecules, tumor necrosis factor receptor (TNFR) super family 1A (TNFRSF1A) or TNFRSF1B[6,7]. The 2 receptors appear to promote mainly distinct cellular responses, yet do share some degree of redundancy in their function, each capable of inducing both the nuclear factor-κB and apoptotic pathways[8-12]. The extent to which TNFR function may vary between cell types is currently unclear, but it is quite evident that the binding of TNF-α to its receptor is imperative for signaling and the initiation of downstream events. Consequently, any conformational changes in either the ligand or the receptor may truncate effective signaling or mediate aberrant signaling.

The levels of soluble TNFR (sTNFR) have been shown to be elevated in the serum and synovial fluid (SF) of RA patients[13,14], while membrane-bound TNFR (mTNFR) are increased on a variety of cells in the RAsynovial tissue[15,16]. Soluble TNFR are derived from the extracellular domain of the membrane bound form and when cleaved retain their ligand binding capacity[17,18]. Therefore sTNFR act as natural inhibitors of TNF-α signaling.

The TNFRSF1A gene maps to 12p13 and consists of 10 exons while TNFRSF1B, also consisting of 10 exons, maps to 1p36[19-22]. Polymorphisms within these receptor genes have been described[23-25] and several studies have analyzed their possible association with RA[26-30]. Two of these studies have reported an association between a specific single nucleotide polymorphism (SNP) in the TNFRSF1B gene and susceptibility to familial RA[26,27]. This SNP occurs at position +196 (T to G) in exon 6 of the gene and lies within the fourth extracellular domain of the folded protein receptor, and results in a nonconservative amino acid substi-

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tution (methionine to arginine). An effect on receptor function has been shown in TNFRSF1B transfected HeLa cells, where functional analysis of the polymorphism revealed both increased interleukin 6 (IL-6) production and increased cytotoxic activity in cells with the +196R TNFR[31]. In addition, it is proposed that the polymorphism may modulate receptor assembly and conformation[31].

While the studies described have provided data on TNFR SNP and RA susceptibility, there has only been one study[32] and an earlier brief report[30] on the possible association of TNFR SNP with RA severity. A recent study by Fabris, *et al*[32] investigated the association between the +196 SNP in TNFRSF1B and disease severity in a Caucasian RApopulation from Southern Europe. Although no statistically significant associations with RA severity were found, they did observe a trend towards an increased frequency of the GG genotype in patients receiving anti-TNF-α treatment because of severe disease. A brief report by Kaijzel, *et al*[30] studied the -383 and +36 polymorphisms in the TNFRSF1A gene, and also a +1690 SNP in TNFRSF1B, but found no associations with disease severity. Our aim was to independently examine the association between TNFRSF1A and TNFRSF1B SNP and disease severity in a different RA population.

MATERIALS AND METHODS

Patients. The association between TNF-α receptor polymorphisms and RA severity was studied in a group of 181 Caucasian RApatients, all of British origin and resident in North Staffordshire, England. The demographic data are displayed in Table 1. All patients had been diagnosed with RA, as classified by the 1987 American College of Rheumatology criteria[33], by a consultant rheumatologist and were receiving antiinflammatory and/or antirheumatic chemotherapy. Most of the patients (> 90%) had been treated with one or more disease modifying antirheumatic drugs (DMARD). The majority of patients were currently being treated with methotrexate (MTX, 52%), sulfasalazine (35%), gold salts (24%), or hydroxychloroquine (18%). The most common combination therapy was MTX and sulfasalazine. A small number of patients were being treated with steroids (5%) or with cytotoxic drugs such as azathioprine or cyclophosphamide (< 5%). Radiographic damage was measured by scoring radiographs of the hands and feet using the method of Larsen, *et al*[34] and functional outcome was assessed by the Health Assessment Questionnaire (HAQ)[35].

Genomic DNAisolation. Fresh peripheral blood samples (about 4 ml) were obtained from each patient and stored in EDTA tubes at –20°C until required. Blood samples were thawed at 37°C and the genomic DNA isolated using a DNAce MegaBlood Kit procedure as directed by the manufacturer (Bioline, Humbar Road, London, England).

Table 1. Characteristics of the patient population with RA.

N	181
Male/female (%)	42.5/57.5
Age, mean ± SD, yrs	57.6 ± 10.9
Age at onset, mean ± SD, yrs	46.3 ± 11.5
Disease duration, mean ± SD, yrs	11.6 ± 5.5
RF* ever positive (%)	67.2
Nodule positive (%)	17.3

* RF: rheumatoid factor

PCR primers. The primer sequences used for amplification of a 183-bp fragment containing the SNPat position +36 in exon 1 of the TNFRSF1Agene were as follows: Forward: 5'- GAG CCC AAA TGG GGG AGT GAG AGG - 3'; and Reverse: 5'- ACC AGG CCC GGG CAG GAG AG - 3'[23].

The primer sequences for the SNP at position +196 in exon 6 of the TNFRSF1B gene were: Forward: 5' - ACTCTC CTA TCC TGC CTG CT - 3'; and Reverse: 5' - TTC TGG AGT TGG CTG CGT GT - 3'[36], and generated a fragment of 242-bp.

Polymerase chain reaction (PCR) amplification. The fragment for amplification from each of the TNF-α receptors was amplified using an identical reaction mixture containing Mg^{2+} (1.25 mM), 10 × buffer, dNTPs (160 µM each), Taq polymerase (1U) and 2 × Poly-mate additive (Bioline); forward and reverse primers (about 6 pmol each; Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, England), DNAand H_2O to a final reaction volume of 25 µl/sample. Wells were then overlaid with mineral oil to prevent evaporation during the amplification protocol.

The amplification conditions for the TNFRSF1ASNPwere: 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 63°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. Amplification products were stored at 4°C until required.

The amplification of the TNFRSF1B SNP was performed under the following conditions: 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. Amplification products were stored at 4°C until required.

TNFRSF1A restriction fragment length polymorphism (RFLP) analysis. The SNPat position +36 in exon 1 of the gene results in the substitution of an adenine base with a guanine (Ato G) and the creation of a recognition site for the restriction endonuclease MspA1 I. PCR products (10 µl) were digested with 2 U MspA1 I (Promega UK, Southampton, UK) at 37°C for 4 h and this generated restriction fragments of 108 and 75 bp when the G allele was present, and an uncut 183 bp fragment when the A allele was present. These restriction fragments were visualized by 4% agarose gel electrophoresis (110 volts, 1 h).

TNFRSF1B RFLPanalysis. The SNPat position +196 in exon 6 of the gene results in the substitution of a thymine base with a guanine (Tto G) and the elimination of a recognition site for the restriction endonuclease Nla III. This substitution represents the G allele and generated a fragment of 242 bp, whereas the T allele gave rise to fragments of 133 and 109 bp, when the PCR products (10 µl) were digested with 2 U Nla III (New England Biolabs, Hertfordshire, England) for 3 hours at 37°C. Restriction fragments were visualized by electrophoresis (110 volts, 1 h) on 4% agarose gels.

Statistical analysis. Associations between the TNF-α receptor SNP and severity of disease, using both the Larsen score and the HAQ score, were assessed using multiple regression analysis with correction for age, sex, and disease duration. All data were analyzed using the Number Cruncher Statistical Software package for Windows (NCSS version 6.0.4). A p value < 0.05 was considered significant.

RESULTS

TNFRSF1A SNP and disease severity. The genotype frequencies for the +36 exon 1 SNP, together with the respective mean Larsen and HAQ scores are shown in Table 2. The observed allele frequencies for the A and G alleles were 56.4% and 43.7%, respectively. Both the allele and genotype frequencies are similar to those reported elsewhere for Caucasian RA/control populations[23,26,30]. There was no significant difference in either measure of disease severity between the 3 genotypes after correction for sex, age, and disease duration (Table 2). There appeared to be an increasing trend in mean severity values from the AAto the rarer GG genotype. However, analysis of trend revealed no

Table 2. Tumor necrosis factor receptor super family 1A (TNFRSF1A) exon 1 single nucleotide polymorphism (SNP) genotype frequencies and RA severity in 181 RA patients, expressed as mean (SD). No significant differences were found between any of the genotypes after correction for age, sex, and disease duration (multiple regression analysis).

Genotype	n (%)	Larsen Score	HAQ Score
AA	53 (29.3)	93.5 (38.4)	1.33 (0.79)
AG	98 (54.1)	95.1 (43.9)	1.53 (0.80)
GG	30 (16.6)	101.8 (49.1)	1.66 (0.93)

HAQ: health assessment questionnaire.

significant evidence of any trend between the genotypes (data not shown).

TNFRSF1B SNP and disease severity. The genotype and disease severity data for the +196 exon 6 SNP are shown in Table 3. The T and G alleles had frequencies of 75.1% and 24.9%, respectively. The allele and genotype frequencies in this population were found to be no different to those previously reported for Caucasian sporadic RA/control populations[26,27]. Analysis revealed no significant differences in either Larsen score or HAQ score between the genotypes, after correction for sex, age, and disease duration (Table 3). Furthermore, trend analysis revealed no significant relationship (data not shown).

DISCUSSION

Polymorphisms in the TNFR genes are ideal candidates for association with disease severity in RA as the receptors mediate the actions of TNF-α, which is heavily implicated in the pathogenesis of RA[1]. Most studies have focused on the role of TNFR polymorphisms in susceptibility to disease[26-30]. These suggest that neither the TNFRSF1A +36 nor the TNFRSF1B +196 SNP are associated with susceptibility to sporadic RA, but the TNFRSF1B polymorphism is associated with development of familial RA[26,27]

Our results do not provide evidence for an association between either SNP in the TNFRSF1Aor TNFRSF1B gene and disease severity in a population of UK Caucasian patients with RA. The data, analyzed using multiple regression with correction for sex, age, and disease duration, failed to reveal any significant associations with either radiographic or functional outcome measures.

Table 3. Tumor necrosis factor receptor super family 1B (TNFRSF1B) exon 6 SNP genotype frequencies and RA severity in 181 RA patients expressed as mean (SD). No significant differences were found between any of the genotypes after correction for age, sex, and disease duration (multiple regression analysis).

Genotype	n (%)	Larsen Score	HAQ Score
TT	104 (57.4)	95.0 (38.5)	1.43 (0.82)
TG	64 (35.4)	95.1 (51.1)	1.56 (0.83)
GG	13 (7.2)	105.1 (37.1)	1.63 (0.86)

The analysis of the SNPat position +196 in exon 6 of the TNFRSF1B gene was undertaken since this polymorphism occurs in the extracellular domain of the receptor, within the fourth cysteine-rich domain (CRD4)[31], and may affect *in vivo* receptor functioning. Indeed, the 4 CRD have been shown to be important for TNF-α binding[37]. However the +196 SNP has been shown to have no significant effect on either receptor binding of TNF-α or on receptor shedding (enzymatic cleavage of the TNFR extracellular domains)[31]. This may explain why no association was found between this polymorphism and disease severity in RA.

The SNP at position +36 in exon 1 of the TNFRSF1A gene does not represent a functional polymorphism, but it is proposed that it may be in linkage disequilibrium with a nearby functional polymorphism[26]. However, no association of this +36 SNP with disease severity was found and this confirms the findings of Kaijzel, *et al*[30]. This would seem to suggest that the +36 SNP, and any polymorphism in linkage disequilibrium with this, do not have any effect on receptor function that influences disease severity.

Although our analysis found no significant association of either SNP with disease severity, there appeared to be a weak trend in the data (see Tables 2 and 3). For each of the TNFR SNP, carriage of the rarer G allele appeared to be associated with higher disease severity scores. Furthermore, there appeared to be an allele dose dependent effect whereby the GG homozygotes display the highest severity scores, followed by the heterozygotes and the homozygotes. In particular, our observation of more severe disease in patients homozygous for the G allele of the +196 TNFRSF1B SNP confirms the observations of Fabris, *et al*[32], who observed a trend towards an increased frequency of GG homozygotes in RA patients receiving anti-TNF-α treatment because of severe disease. Together, these observations may indicate the existence of a weak association between the SNPat position +196 in exon 6 of the TNFRSF1B gene and disease severity in RA. Additional studies in other populations are required to confirm this trend.

In conclusion, our results do not show an association between either of 2 TNFR SNP and disease severity in RA. These findings confirm those of an earlier preliminary study in Caucasians[30]. However, the possibility of a trend towards more severe disease in GG homozygotes for these TNFR SNP cannot be ruled out. A recent study by Bridges, *et al* reported significant racial/ethnic differences in TNFR SNP frequencies[28], and together with involvement of the TNFRSF1B +196 SNP in susceptibility to familial RA[26,27], provides evidence for genetic heterogeneity. Thus further investigations are needed on other ethnic populations and family subjects to assess the role of these and other TNFR polymorphisms in the pathogenesis of RA.

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