Genetic analysis of the 3’ untranslated region of the tumour necrosis factor shows a highly conserved region in rheumatoid arthritis affected and unaffected subjects

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Abstract

Tumour necrosis factor (TNF) is a key proinflammatory mediator in rheumatoid arthritis (RA). The TNF locus, situated in the class III region of the MHC, is flanked by five microsatellite markers. It has previously been shown that this region influences susceptibility to RA; two TNF microsatellite haplotypes were found to be associated with RA. Evidence from murine studies has indicated that variation in the TNF 3’ untranslated region (UTR) could be associated with altered regulation of TNF biosynthesis. In order to identify possible RA associated polymorphisms, more than 800 bp of the TNF 3’ UTR was genetically analysed in RA affected and unaffected subjects possessing specific RA and non-RA associated TNF microsatellite haplotypes. The TNF 3’ UTR region was analysed using two mutation detection methods, PCR-SSCP and NIRCA analysis and DNA sequencing.

No genetic differences were observed in the human TNF 3’ UTR between subjects, that is, irrespective of RA status or TNF haplotype, and also compared with previously published TNF sequences from human sources. Therefore it can be concluded that the TNF 3’ UTR in this population was highly conserved and did not influence susceptibility to RA.

Keywords: autoimmunity; MHC class III; regulatory sequences; gene polymorphism

The aetiology of rheumatoid arthritis (RA) is largely unknown, but both genetic and environmental factors are involved in RA susceptibility. A consistent finding in genetic studies of RA is an association with conserved amino acid motifs, termed the “shared epitope” (SE), in the third hypervariable region of the HLA-DRB1 molecule, located in the class II region of the major histocompatibility complex (MHC). Recently it has also been shown that the TNF-lymphotoxin (LT) locus, located in the class III region of the MHC, influences susceptibility to RA, distinct from the SE. The inheritance of five TNF microsatellite markers flanking the TNF-LT locus was studied in 50 multicase RA families. From 48 TNF microsatellite haplotypes defined, two were found to be associated with RA susceptibility; the TNF a6, b5, c1, d3, e3 (H1) haplotype in homozygotes for the SE and the TNF a2, b3, c1, d1, e3 (H2) haplotype in heterozygotes for the SE. This association suggests that a distinct genetic element located in the class III region of the MHC, possibly the TNF gene itself, plays a role in susceptibility to RA.

Post-transcriptional regulation of TNF is achieved through the 3’ untranslated region (UTR) where AU rich sequence motifs are believed to act in concert with their flanking regions to effect TNF mRNA stability, thus mediating translational control. The murine TNF 3’ UTR is highly polymorphic and in vitro studies showed that regulatory polymorphisms located in this region had a significant effect on TNF levels. Transgenic mice whose TNF gene was modified to carry a human TNF 3’ region developed a polyarthritis similar to RA which was prevented by treatment with...
monoclonal antibodies to human TNF. The data suggested that putative polymorphisms located in the human TNF 3' UTR could potentially influence TNF production leading to the development of autoimmune diseases. In order to elucidate if the human TNF 3' UTR specifically plays a role in disease susceptibility, a population of RA affected and unaffected subjects carrying defined TNF haplotypes was genetically analysed to investigate for possible polymorphism(s).

Methods
A panel of 38 subjects carrying in total 19 different TNF haplotypes was selected for study on the basis of possession of specific TNF haplotypes, RA status, and HLA-DRB1 genotype (table 1). The members of the panel had been participants in a previous study investigating the role of TNF in RA susceptibility. DNA isolation, TNF microsatellite analysis, and HLA-DRB1 typing protocols were performed as previously described.

A primary PCR amplification of the TNF 3' UTR using primers T11 and T16 (fig 1A), designed to the previously published TNF sequence, was performed to generate a 924 bp product. PCR conditions were as described previously with an annealing temperature of 55°C and a final cycle of 94°C for one minute, 55°C for one minute, and 72°C for five minutes. For SSCP analysis secondary PCR using the primary product as template and incorporating 10 µCi of [dATP^35S] 500 Ci/mmol was used for the assay. (B) NIRCA analysis of the TNF 3' UTR experimental sense strands of RNA hybridised to an antisense control RNA strand. Lanes 1-9: subjects 10, 6, 7, 13, 4, 8, 6, 7, and 27 respectively. Lane 10: subject 3, control.
mmol (New England Nuclear, Du Pont, Boston, MA), was carried out to generate three overlapping segments using specific primer pairs (fig 1A). PCR-SSCP analysis was carried out as previously described.11,12

For the non-isotopic RNase cleavage assay (NIRCA) (Ambion, Austin, Texas) a primary template was amplified as for SSCP using 500 ng of genomic DNA, 2.5 units of Taq polymerase, and a biotinylated T11 primer. Streptavidin coated dynabeads (Dynal (UK) Ltd, Wirral, UK) were used to purify PCR products. The 642 bp fragment encompassing the core “AU” repeat was amplified using a sense primer, MD1 (containing a T7 promoter), and an antisense primer, MD2 (containing an Sp-6 promoter) (fig 1A). NIRCA analysis was carried out according to the manufacturer’s protocol using control transcripts from three subjects: No 3, homozygous for TNF haplotype H1, No 9, homozygous for TNF haplotype H2, and No 21, homozygous for TNF haplotype H7. The primary PCR product was purified with QIAquick PCR purification kit (Qiagen Ltd, Wirral, UK) and an ABI PRISM Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, UK) and an ABI PRISM 310 Genetic Analyser (Perkin-Elmer, Warrington, UK).

Results and discussion

Data from murine studies indicated that polymorphisms found in the TNF 3’ UTR region could cause altered regulation of TNF biosynthesis.7 In order to investigate this in humans, the TNF 3’ UTR was analysed to determine if polymorphism(s) were present. PCR-SSCP and NIRCA were carried out on samples from 33 subjects (23 RA affected, 10 unaffected). In total, 18 distinct TNF haplotypes were examined (table 1). No altered mobility of single stranded DNA (ssDNA) from any subject was observed on PCR-SSCP analysis. When NIRCA was applied, identical RNase cleavage multibanding patterns were observed for all subjects studied (fig 1B). The multibanding pattern was most likely because of cleavage at AU rich regions which are hypersensitive to RNase digestion.

The entire TNF 3’ UTR was sequenced in both orientations (813 bp) in 12 subjects (eight RA affected, four unaffected) carrying in total nine TNF haplotypes (table 1). These sequences were compared between subjects using Megalign software (DNASTAR, Madison, WI, USA) and with previously documented TNF 3’ UTR sequences present in non-redundant databases using the Blastn search program.13 There was no difference in the sequence of the TNF 3’ UTR either between subjects or in comparison with previously documented TNF 3’ UTR from human sources. DNA sequence analysis of the TNF 3’ UTR confirmed the results from the two mutation screening methods indicating that no polymorphism(s) occurred. It would appear then that the TNF 3’ UTR is highly conserved in these subjects despite differing RA status, HLA-DRB1 type, and TNF haplotype. Therefore, the previous published associations of TNF haplotypes H1 and H2 with RA susceptibility7 have not been explained by this study. It is possible that the association of the TNF haplotypes with RA is the result of altered transcriptional regulation of the TNF gene or variants of another gene elsewhere in the class III MHC.

The results from this study concur with a recent study by Becker et al,14 where no polymorphisms were found in the TNF 3’ UTR in patients with connective tissue disease or type I diabetes. Therefore, in contrast to the highly polymorphic murine TNF 3’ UTR, the human TNF 3’ UTR appears to be highly conserved in these populations.

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