R. Ehling
Ch. Gassner
A. Lutterotti
S. Strasser-Fuchs
H. Kollegger
W. Kristoferitsch
M. Reindl
T. Berger

Genetic variants in the tumor necrosis factor receptor II gene in patients with multiple sclerosis

Key words:
MS; polymorphism; susceptibility; TNFR II

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Abstract: Common genetic variants have been shown to influence disease susceptibility, disease course, or both in multiple sclerosis (MS). Several studies have suggested a role for tumor necrosis factor-α (TNF-α) in the pathogenesis of MS. Recently, it has been reported that the TNF receptor (TNFR) II plays an essential role in the pathology and progression of experimental autoimmune encephalomyelitis, an animal model of MS. To investigate whether TNFR II polymorphisms influence susceptibility and/or clinical progression of MS, genomic DNA of 321 samples of the Austrian Genetics in MS study group and DNA of 174 platelet donors, who served as healthy controls, were genotyped for five polymorphic sites in the *TNFR II* gene: *exon 6 nucleotide (nt) 676*T→G*, *exon 6 nt 783*G→A* (both are associated with non-conserved amino acid substitution), *exon 10 nt 1663*G→A*, *exon 10 nt 1668*T→G*, and *exon 10 nt 1690*T→C* (all of which are located in the 3′ non-coding region of the gene). We found a significant association between *exon 10 nt 1668*T→G* polymorphism and susceptibility to MS. The other investigated nucleotide substitutions were not associated with susceptibility to or clinical parameters in MS.

Authors' affiliations:

R. Ehling[1]*,
Ch. Gassner[2]* ,
A. Lutterotti,
S. Strasser-Fuchs[3],
H. Kollegger[4] ,
W. Kristoferitsch[5],
M. Reindl[1],
T. Berger[1]

[1]Department of Neurology, University of Innsbruck, Anichstrasse, Innsbruck, Austria,

[2]Central Institute for Blood Transfusion and Immunological Department, University Clinics, Anichstrasse, Innsbruck, Austria,

[3]Department of Neurology, University of Graz, Auenbruggerplatz, Graz, Austria,

[4]Department of Neurology, University of Vienna, Währinger Gürtel, Vienna, Austria,

[5]Department of Neurology, SMZ Ost, Langobardenstrasse, Vienna, Austria

Correspondence to:
T. Berger, MD
Department of Neurology
University of Innsbruck
Anichstrasse 35, A-6020
Innsbruck, Austria
Tel.: +43-512-504-3860
Fax: +43-512-504-4260
e-mail: thomas.berger@uibk.ac.at

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Multiple sclerosis (MS) is believed to be an inflammatory autoimmune disorder of the central nervous system (CNS). Several studies have indicated that tumor necrosis factor-α (TNF-α), a proinflammatory cytotoxic cytokine, is a critical immunopathogenetic mediator in MS and its animal model experimental allergic encephalomyelitis (EAE) (1–3). TNF-α induces a wide variety of responses including apoptotic cell death, enhanced ischaemic and excitotoxic injury in neurons, but also neuroprotection (4–6). The coexistence of neurotoxic and neuroprotective effects of TNF-α may be partially explained by two different signaling pathways mediated by two cell-surface TNF receptors (TNFR) with 55 kDa (TNFR I) and 75 kDa (TNFR II) (7, 8). *In vivo* studies have demonstrated that TNFR I mainly mediates CNS inflammation and demyelination,

*Both the authors contributed equally to this work.

whereas TNFR II enhances remyelination (9, 10). Both the receptors have also been shown to exist in soluble forms and are thereby able to neutralize circulating TNF-α (11). Serum and cerebrospinal fluid concentrations of the soluble forms of both the receptors have been shown to correlate with clinical activity in MS patients (12, 13).

Therefore, genetic association studies have been performed to further study the role of TNFR I and TNFR II in MS. In one study, no association between microsatellite markers in the *TNFR I* and *TNFR II* gene with MS has been found (14). Another study, investigating polymorphisms in the *TNFR I* gene in MS patients using restriction enzyme digests, found almost no genetic variation in the coding region and no association with disease course or severity (15). Yet, TNFR II, located on chromosome 1p36.3-p36.2, shows a certain degree of allelism. Biallelic polymorphisms leading to amino acid substitutions have been identified in *exon 4*, *exon 6*, and *exon 9*, non-coding single-nucleotide polymorphisms (SNPs) have been found in *exon 10* of the *TNFR II* gene (16, 17). Among these polymorphisms, the *G* allele of SNPs at *nt 676*, located in *exon 6*, which encodes parts of the transmembrane region and a proteolytic cleavage site that produces soluble TNFR II (18), has been shown to be associated with susceptibility and disease course in other autoimmune disorders, such as systemic lupus erythematosus (SLE) (17, 19, 20) and rheumatoid arthritis (RA) (21–23). The associations, however, were not present in patients from Korea (24), Spain, or UK (25), possibly due to different frequencies of the respective allele in these populations (26). The findings in other autoimmune diseases and the implications of TNF-α and the potential role of its receptors TNFR I and TNFR II in the pathogenesis of MS and EAE encouraged us to investigate whether genetic variants of the *TNFR II* gene are associated with susceptibility and clinical progression in MS.

Materials and methods

Study population

Clinical and genetic data of 321 patients from the Austrian Genetics in MS study group were collected in four major outpatient clinics for MS in Austria (27, 28). All patients were unrelated Austrian Caucasians, fulfilled the criteria of Poser (29) and had a disease duration of at least 3 years. Disability was scored using the expanded disability status scale (EDSS) (30). Patients were classified as having primary progressive (PP) or bout onset (BO) MS. As a group of geographically comparable healthy controls, we included 174 anonymized platelet donors (107 males and 67 females, mean age 26.0 years, standard deviation 9.4 years) from the General Hospital and University Clinics (Innsbruck, Austria). Demographic and clinical data of MS patients are summarized in Table 1.

Polymerase chain reaction using sequence-specific primer technique (PCR-SSP)

GenBank (GenBank, National Center for Biology Information resources page) was searched with a sequence representative of the *TNFR II* gene (18, 31) using the BLAST program (Basic Logical Alignment Search Tool, available at: http://www.ncbi.nlm.nih.gov/BLAST). Genomic DNA was purified from peripheral blood leukocytes using a nucleon extraction and purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK), and PCR-SSP was performed on each extracted DNA. In each PCR, control human growth hormone oligonucleotides were used to amplify a 434-bp PCR fragment from the human growth hormone locus position 5559 to 5992 (32), which served as a positive amplification control. All primers were synthesized at Microsynth (Balgach, Switzerland) and their sequences are summarized in Table 2.

PCR was performed in a final volume of 10 µl, containing 60 ng genomic DNA as template (quantified by ultraviolet light), 10 mM Tris–HCl, 50 nM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1 mg/ml cresol red, 5.0% glycerin and 0.4 U *Taq* polymerase (Perkin Elmer, Wellesley, MA, USA). To facilitate TNFR II DNA typing, all 11 PCRs were triggered to work under the same thermocycling conditions on a DNA thermal cycler (PCR System 9600, Perkin Elmer). Amplification conditions consisted of initial denaturation of 120 s at 94°C, 10 incubation cycles for 10 s at 94°C and 60 s at 65°C, followed by 20 incubation cycles for 10 s at 94°C, 50 s at 61°C, and 30 s at 72°C. PCR fragments were separated by size in a 2% agarose gel containing 0.5 µg/ml ethidium bromide, visualized with ultraviolet light, and

Clinical data of multiple sclerosis (MS) patients

	BO ($n=300$)	PP ($n=21$)	All MS ($n=321$)
Females	212 (70.7%)	11 (52.4%)	223 (69.5%)
Age[a]	40.1 (33–46)	52.5 (40–63)	40.9 (33–47)
Age at onset[a]	27.2 (22–32)	41.1 (28–52)	28.1 (22–33)
Disease duration[a]	13.0 (6–18)	11.4 (6–13)	12.9 (6–18)
EDSS[a]	3.4 (1.5–4.8)	5.6 (4.3–6.8)	3.6 (1.5–5.5)
Progression index[a,b]	0.3 (0.2–0.4)	0.7 (0.4–0.8)	0.4 (0.2–0.5)
Relapses[a]	6.5 (4.0–8.0)		
Annual relapse rate[a]	0.8 (0.4–1.1)		

BO, bout onset MS; EDSS, expanded disability status scale; PP, primary progressive MS.
[a]Medians (interquartile range).
[b]Defined as EDSS/disease duration in years.

Table 1

Primers used in this study

Name	Nucleotide sequence (5′–3′)
TNFR II+676FG	GGCCATCCCTGGGAATGCAAGAA*G*
TNFR II+676FT	GGCCATCCCTGGGAATGCAAGAA*T*
TNFR II+677FT	GGCCATCCCTGGGAATGCAAGCC*T*G
TNFR II+782RA	TGCTTGGAGCAGTGCTGGGTAT*T*
TNFR II+783RG	GGTGCTTGGAGCAGTGCTGGGGT*C*
TNFR II+1663FA	GCCAAGAGCAGAGGCAGGG*A*
TNFR II+1663FG	CCAAGAGCAGAGGCAGCG*G*
TNFR II+1668FG	AGAGCAGAGGCAGCGCGTTG*G*
TNFR II+1668FT	AAGAGCAGAGGCAGCGCGTTG*T*
TNFR II+1690GR	CAGCCTTCCGAGAGGGACCC*G*
TNFR II+1690TR	CAGCCTTCCGAGAGGGACGC*A*
TNFR II+1909R	GCATCCCTCCTCTCCAGAAAGCC
TNFR II-i5-76F	actctcctatcctgcctgctggg
TNFR II-i6+53R	aaaccatgggagaggaagaaagagagac
TNFR II-i9-119F	agcagagctgggctagaatcttgg
HGH-352F	TGCCTTCCCAACCATTCCCTTA
HGH-739R	CCACTCACGGATTTCTGTTGTGTTTC

F, forward; R, reverse; i, primer located in intron; number represents the distance of the 3′ end of the primer to the next exon; intronic primers are given in lower-case letters. Specificities are given in italicized letters. The number of all other primers refers to the location of the 3′ end of the primer on TNFR II mRNA. Primers for human growth hormone (HGH) were used as controls for amplification in all PCR-SSPs.

Table 2

photodocumented. All polymorphisms were confirmed in selected cases by direct sequencing (Microsynth), and variants of potential interest were partly screened using restriction fragment length polymorphism (RFLP) of PCR-amplified sequences.

Human leukocyte antigen typing was performed for class II genes using PCR amplification with sequence-specific primers in a low-resolution kit (Olerup SSP-Combi Tray, Saltsjöbaden, Sweden). PCR products were analyzed by agarose gel electrophoresis and interpreted using the HELMBERG SCORE Software (Geno Vision, West Chester, PA, USA).

Statistical analysis

Statistical analysis (frequencies, means, standard deviations and medians) and significance of group differences (Fisher's exact test, Chi-square test) were performed using the GRAPHPAD PRISM statistical analysis program (GraphPad Software, San Diego, CA, USA). After Bonferroni's correction for three comparisons, P-values < 0.017 were considered to indicate statistical significance. For two comparisons, P-values < 0.05 were considered to indicate statistical significance.

Results

One of the main results of this study was the detection of an SNP in *exon 6 nt 783*G→A* for the first time in a Caucasian population, which results in a non-conserved amino acid substitution: Glutamic acid at 783 (GAA) is substituted by lysine (AAA). In addition, we confirmed known SNPs: *exon 6 nt 676*T→G*, resulting in a substitution of methionine by arginine, *exon 10 nt 1663*G→A*, *exon 10 nt 1668*T→G*, and *exon 10 nt 1690*T→C* (all the three do not cause amino acid substitutions, because they are located approximately 200 bp downstream of the stop codon). Figure 1 shows SNP-PCR results of two representative TNFR II genotypes. Allele frequencies and genotype distribution at the individual loci are summarized in Table 3. The frequencies of all five biallelic polymorphisms confirmed to the Hardy–Weinberg equilibrium constant. The observed frequencies of *exon 6 nt 676*T→G* and of the three polymorphic sites in *exon* 10 are consistent with the previously found genotype distributions in healthy Caucasians (16, 21, 26). All four possible haplotypic combinations of the two *exon 6* SNPs could be typed individually. This was done using two monospecific PCR-SSPs for *exon 6 nt 676*T* and **G*, one monospecific PCR-SSP for *exon 6 nt 783*A* and two bispecific PCR-SSPs, one detecting a cis presence of *676*T-783*G*, the other a cis presence of *676*G-783*G*. The most common haplotype was found to be *exon 6 nt 676*T/exon 6 nt 783*G*, in both MS patients and controls (74.7% *vs* 78.5%; $P = 0.21$). Only the *G* allele of *exon 6 nt 676* was found to be associated with *exon 6 nt 783*A*.

There were no significant differences in allele frequencies and genotype distribution of SNPs in the *TNFR II* gene between MS patients and controls, with the exception of *exon 10 nt 1668*T→G* polymorphism. In MS patients, 2.5% were positive for the *1668*G* allele, whereas 5.5% were positive in the healthy control group ($P = 0.019$). Genotype analysis revealed that the lower frequency of the *1668*G* allele in MS patients was caused by an increase in the proportion of *1668*T/T* homozygotes ($P = 0.048$).

We found a highly significant association between the HLA DQB1*06, DRB1*15, and DRB5 alleles and the presence of MS (all $P < 0.001$) (28, 33). One-hundred and thirty one MS patients were stratified on the basis of HLA DR15 genotype and correlated to *exon 10 nt 1668*T→G* polymorphism. Patients who were positive for HLA DRB1*1501 did not differ in their genotype distribution from those who were negative ($P = 0.19$).

When relapsing–remitting and secondary progressive MS patients were compared to those with PP MS, no differences in genotype and phenotype frequencies were found. We further investigated whether one of the polymorphisms had an influence on clinical parameters, such as disease duration, relapse rate and progression

Allele frequencies and genotype distribution

Locus	Allele	MS (%)	Control (%)	*P*-value	Genotype	MS (%)	Control (%)	*P*-value
exon 6 nt 676	*T*	480 (74.8)	273 (78.4)	NS	*TT*	178 (55.5)	103 (59.2)	NS
	G	162 (25.2)	75 (21.6)		*TG*	124 (38.6)	67 (38.5)	
					GG	19 (5.9)	4 (2.3)	
exon 6 nt 783	*G*	629 (97.8)	336 (96.6)	NS	*GG*	308(96.0)	163 (93.7)	NS
	A	13 (2.0)	12 (3.4)		*GA*	13 (4.0)	10 (5.8)	
					AA	0(0.0)	1(0.6)	
exon 10 nt 1663	*G*	354 (55.1)	184 (52.9)	NS	*GG*	104 (32.4)	48 (27.6)	NS
	A	288 (44.9)	164 (47.1)		*GA*	146 (45.5)	88 (50.6)	
					AA	71 (22.1)	38 (21.8)	
exon 10 nt 1668	*T*	626 (97.5)	329 (94.5)	0.019	*TT*	305 (95.0)	156 (89.7)	NS
	G	16 (2.5)	19 (5.5)		*TG*	16 (5.0)	17 (9.8)	
					GG	0 (0.0)	1 (0.6)	
exon 10 nt 1690	*T*	398 (62.0)	224 (64.4)	NS	*TT*	121 (37.7)	72 (41.4)	NS
	C	244 (38.0)	130 (35.6)		*TC*	156 (48.6)	80 (46.0)	
					CC	44 (13.7)	22 (12.6)	

P-values were determined using Fisher's exact test or chi-square test. After Bonferroni's correction for three comparisons, *P*-values of less than 0.017 were considered to indicate statistical significance. MS, multiple sclerosis.

Table 3

Reaction number	1	2	3	4	5	6	7	8	9	10	11
Product in bp	304	304	260	151	151	289	289	284	284	658	658
Kind of specificity	Mono	Mono	Mono	Bi	Bi	Mono	Mono	Mono	Mono	Mono	Mono
Specificity	*676*G*	*676*T*	*783*A*	*676*T-783*G*	*676*G-783*G*	*1663*G*	*1663*A*	*1668*T*	*1668*G*	*1690*T*	*1690*C*
676*G-783*G	+	–	–	–	+						
676*T-783*G	–	+	–	+	–						
676*G-783*A	+	–	+	–	–						
676*T-783*A	–	+	+	–	–						
1663*G						+	–				
1663*A						–	+				
1668*T								+	–		
1668*G								–	+		
1690*T										+	–
1690*C										–	+

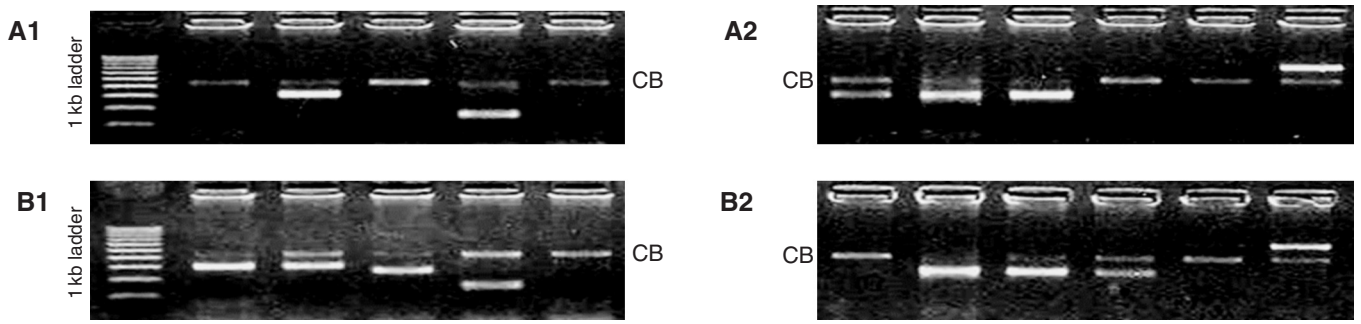

Fig. 1. Tumor necrosis factor receptor (TNFR) II polymerase chain reaction-sequence-specific primers (PCR-SSP) results of two representative genotypes. Panels A1 to B2 show agarose gel electrophoresis of 11 performed TNFR II PCR-SSPs. The TNFR II PCR-SSP characteristics are shown in the interpretation scheme at the top of the figure. CB, control band of human growth hormone with 434 bp. Panel A1 and A2 show homozygosity in *exon 6 nt 676*T* and *exon 6 nt 783*G*, heterozygosity in *exon 10 nt 1663*G/A* and homozygosity in *exon 10 nt 1668*T* and *exon 10 nt 1690*C*. In addition, panel A1 shows the most common haplotype: homozygosity in *exon 6 nt 676*T/exon 6 nt 783*G*. Panel B1 and B2 show heterozygosity in *exon 6 nt 676*T/G*, *exon 6 nt 783*G/A*, *exon 10 nt 1663*G/A*, and *exon 10 nt 1690*T/C*, homozygosity in *exon 10 nt 1668*T/G*. Panel B1 refers to the rare haplotypes: *exon 6 nt 676*T/exon 6 nt 783*G* and *exon 6 nt 676*G/exon 6 nt 783*A*.

rate (EDSS/disease duration in years). There was no association between genotype and carrier status of any polymorphism and gender, age, age at onset, EDSS, disease duration, and number of relapses in MS.

Discussion

We developed a PCR-SSP system to examine two biallelic polymorphisms in *exon 6* and three in *exon 10* of the *TNFR II* gene on the basis of variations between published sequences. *Exon 6 nt 676*T→G* mutation was found at frequencies comparable to that in healthy individuals from UK (16, 21), Spain (25), and USA (25, 26). The frequencies of *exon 10 nt 1663*G→A*, *exon 10 nt 1668*T→G*, and *exon 10 nt 1690*T→C* mutations were similar to those described for the population of UK (16). The allele frequency for *exon 6 nt 783*G→A* polymorphism, which is described here for the first time in a European population, does not differ from that reported in a Japanese population (17). In contrast to the findings for *exon 6 nt 676*T→G* mutation (26), racial and ethnic differences do not appear to influence *exon 6 nt 783*G→A* mutation.

An association of *exon 6 nt 676*T→G* polymorphism with susceptibility and disease activity has been described for other autoimmune diseases such as SLE and RA (17, 19, 20, 22), particularly familial forms of those disorders (21, 34). We were therefore interested whether these associations found in SLE and RA are also observed in MS. We found similar allele frequencies and genotype distributions for *exon 6 nt 676*T→G*, *exon 6 nt 783*G→A*, *exon 10 nt 1663*G→A*, and *exon 10 nt 1690*T→C* mutation in MS patients from Austria and in a healthy control population from a comparable geographic region. Significant differences were found in the genotype distribution of *exon 10 nt 1668*T→G* polymorphism between MS patients and the healthy control group. In view of the very low frequency of the *G* allele and the intronic location, these findings should be interpreted with caution. However, the differences in the *exon 10 nt 1668*T→G* polymorphism between patients and controls might indicate that the TNFR II locus partly contributes to overall MS susceptibility independent of HLA loci. Because females, as in other studies on autoimmune disorders, were over-represented in our MS study population (69.5%), we did a covariate analysis on gender, which revealed no differences in polymorphism distributions. The same was true for age, and we therefore believe a younger healthy control group compared to the MS population to be justified.

We further analyzed whether MS patients with certain genotypes have an altered MS disease course. For this purpose, we compared disease subtypes (RR and BO) and clinical parameters among the different TNFR II alleles and carrier status. We, however, did not find any disease modifying the influence of one of the investigated TNFR II alleles on MS.

Attempts to find positive correlations between the distribution of *TNFR II* gene variants and clinical parameters such as MS course, annual relapse rate, and progression index failed. Thus, TNFR II polymorphism appears to contribute to the susceptibility to MS but not to its phenotype or clinical severity.

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