

A link between promoter polymorphisms of the *transforming growth factor β1 (TGFB1)* and *TGF-β1 receptor II (TGFB2)* genes and relapsing-remitting multiple sclerosis

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Abstract

Introduction: Multiple sclerosis (MS) is a chronic progressive autoimmune disease characterised by nerve demyelination, mediated by myelin-specific Th1 autoreactive cells. Transforming growth factor β1 (TGF-β1) is a regulatory cytokine involved in MS aetiology by maintaining CD4+ cell differentiation and preventing autoimmune responses. Because of the important role of the TGF-β1 signalling pathway in MS aetiopathogenesis, we aimed to investigate the association of two DNA polymorphisms: TGFB1C[-509]T and TGFB2G[-875]A and their combined genotypes with the risk of MS development in a cohort of Bulgarian patients. The effect of the two promoter polymorphisms on the disease onset was also assessed.

Material and methods: In the study, a cohort of 183 patients with relapsing-remitting multiple sclerosis (RRMS) and 307 sex- and age-matched healthy subjects were recruited. Genotyping of the TGFB1C[-509]T (rs1800469) and TGFB2G[-875]A (rs3087465) polymorphisms was performed by PCR-RFLP and PIRA-PCR approaches.

Results: Frequencies of the TGFB1T[-509]T genotype and TGFB1[-509]*T-allele were lower in RRMS men than in control healthy men (15.7% vs. 26.9%, 37.3% vs. 50.7%, respectively). Among males, the TGFB1T[-509]T genotype was related to a significantly reduced risk of RRMS (OR = 0.360, 95% CI: 0.126-1.028, $p = 0.05$) in comparison to the TGFB1C[-509]C genotype. Also, TGFB1[-509]*T-allele was more common in men with RRMS than in healthy men relative to the TGFB1[-509]*C-allele and was associated with a statistically significant protective effect (OR = 0.576, 95% CI: 0.341-0.974, $p = 0.039$). The combination of TGFB1T[-509]T/TGFB1T[-509]C and TGFB2G[-875]A genotypes among men was associated with a significant protective effect compared to the wild-type homozygous TGFB1C[-509]C and TGFB2G[-875]G genotypes (OR = 0.268, 95% CI: 0.088-0.818, $p = 0.018$). No significant association between rs1800469 and rs3087465 was observed among females with and without (controls) RRMS.

Conclusions: In summary, we suggest that in males, a higher TGF-β1 level determined by TGFB1T[-509]T genotype in combination with the TGFB2G[-875]A genotype might be a protective factor against RRMS development.

Key words: multiple sclerosis (MS), cytokine, TGFB1 gene, TGFB2 gene, SNP.

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease characterised by demyelination and neurodegeneration in the central nervous system (CNS), mainly affecting women. Immune abnormalities include both the presence of activated autoreactive T cells and the autoantibodies against myelin, which suggest that defects in immune system regulation contribute to the pathogenesis of the disease [2,12]. Relapsing-remitting MS (RRMS) is described by unpredictable relapses followed by a period of improvement or stable clinical condition (remission), lasting at least a month [1]. Underlying events include disturbance in the tolerance to self-antigens, particularly myelin basic protein, and triggering of the severe autoimmune process, which progresses when left untreated, leading to nerve degeneration. It is well-known that thymus-derived natural T regulatory (Tregs) cells (CD4+CD25+FoxP3+) are a major player in maintaining self-tolerance and preventing autoimmune responses. Experiments with animal models clearly show that the depletion of Tregs causes induction of severe autoimmune conditions of the CNS [21]. Several studies provide evidence that in MS, the suppressive role of Tregs is diminished due to reduced suppressive capacity, and diminished number or restricted T-cell receptor (TCR) repertoire of CD4+CD25+FoxP3+ cells [8,14,34]. The molecular mechanism of Tregs negative control on immune response includes the production of the transforming growth factor β 1 (TGF- β 1), activation of intracellular TGF- β 1 signalling pathway, and expression of Foxp3 transcription factor [19]. Loss of TGF- β 1 in knockout mice resulted in the development of multifocal inflammatory disease [32]. A dominant-negative TGF- β receptor type II mouse model also developed autoimmune disease [4].

Alterations of TGF- β 1 expression, the immunosuppressive cytokine, have been reported in MS. But it remains not fully defined whether the reduced level of TGF- β or changed TGF- β signal transduction pathway contributes to the pathogenesis of the disease [7,34]. Therefore, TGF- β 1 is one of the most extensively studied cytokines, regarding the immunopathogenesis of RRMS [16]. TGF- β 1 is a cytokine with regulatory activities that orchestrate the differentiation of both Tregs and T helper 17 (Th17) cells in a concentration-dependent manner – low doses induce Th17 cell differentiation, while high doses

inhibit Th17 cell development and promote Tregs [25]. In MS TGF- β 1 plays a negative role in the differentiation of encephalitogenic Th17 cells and diminishes the encephalitogenic capacity of myelin-specific Th1 cells by inducing robust production of interleukin 10 (IL-10) in an SMAD4-dependent manner. The SMAD4 (SMAD family member 4 gene) protein is a central mediator of the TGF- β canonical signalling pathway. This protein forms homomeric or heteromeric complexes with other activated Smad proteins, which then accumulate in the nucleus and serve as the transcription factors that regulate the expression of target genes leading to enhancement of Th1 and Th17 response [10,17,36].

The effect of TGF- β 1 on target cells is mediated by two receptors: TGFBR1 and TGFBR2. TGF- β 1 binds to the TGFBR2, which recruits a type I receptor dimer to create a hetero-tetrameric complex that induces SMAD-dependent or SMAD-independent intracellular signalling pathway [31]. TGF- β signalling is altered in MS, and the modulation of this intracellular pathway may represent a potential target for therapy [23,24]. The inability of Tregs in patients with MS to suppress autoimmunity could be attributed to decreased TGFBR2 expression and/or disturbance in the intracellular transducing pathway. In a recent study, it was shown that TGFBR2 expression was reduced in CD4+ T-cells of RRMS patients [22]. In view of the importance of the TGF- β 1 signalling pathway in the aetiology of MS, our understanding of how genetic polymorphisms located in the TGF- β 1 gene and in genes for TGF- β 1 receptors influence MS development seems to be important.

The *TGFB1* gene is located on chromosome 19 in the 19q13.2 region, and several polymorphisms have been identified in the gene, including a promoter polymorphism at -509 position (rs1800469), which is due to a C-to-T transition (TGFB1C[-509]T) and strongly affects the level of TGF- β 1 expression [30]. The *TGFB2* gene located on chromosome 1 in the 1q41 region and the polymorphic variant in the promoter region of *TGFB2* (rs3087465), causing a G-to-A transition, was reported by Seijo *et al.* and was not explored previously as a genetic risk factor for RRMS [28].

Because of the important role of TGF- β 1 signalling pathway in the development of MS, we aimed to investigate the association between two promoter polymorphisms in the two different genes: TGF- β 1C[-509]T and TGFBR2G[-875]A and/or a combina-

tion of the observed genotypes with a risk of MS in a cohort of Bulgarian patients using a case-control gene-association study approach. We also calculated the combined effect of the two promoter polymorphisms on the onset of the disease.

Material and methods

Patients and controls

This study included a group of 183 patients, consisting of 132 women and 51 men with RRMS. The disease type was defined as relapsing-remitting, according to McDonald's criteria [26]. Patients' disability was estimated by using the Expanded Disability Status Scale (EDSS) [15]. All patients were between 18 and 60 years of age and were in a remission stage of the disease (defined as a period of improvement or stable clinical condition for at least 3 months). With regard to disease-modifying therapy, 80.3% of the patients were treated with interferon β (IFN- β), 14.2% were treated with glatiramer acetate for at least six months, and 5.5% were without treatment. All patients were with an EDSS score in the range 1.0 to 4.0. According to the onset of the disease, the group of RRMS patients was separated into two subgroups: early-onset – under 30 years (51.9%) and late-onset – above 30 years (48.1%). All patients were recruited from the Department of Neurology, Medical University-Plovdiv. The clinical characteristics of the RRMS patients are presented in Table I. The control group consisted of 307 healthy volunteers, matched with patients by age and gender. The healthy controls included 240 females and 67 males, with a mean (\pm SD) age of 42.28 \pm 13.17 years.

All cases and controls were Caucasians. The involvement of participants in the study was certified by written informed consent, according to the ethical guidelines of the Helsinki Declaration.

Genotyping of TGFB1C[-509]T and TGFB2G[-875]A promoter polymorphisms

Genomic DNA from peripheral blood samples was extracted using Gene Matrix Purification Kit (EURx, Poland) following the manufacturer's instructions.

Genotyping of the TGFB1C[-509]T (rs1800469) was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, as described in detail previously [33]. The set

of primers used for amplification of 153bp fragment were as follows: forward primer 5'-CAGTAAATGTATGGGTCGAG-3' and reverse primer 5'-GGTGTCACTGGGAGGAGGG-3'. The conditions of the PCR reaction were initial incubation of samples for 3 min at 94°C, and after that 30 cycles as follows: denaturation for 45 s at 94°C, annealing for 45 s at 63.3°C, and extension for 45 s at 72°C; and final extension for 7 min at 72°C. The amplicons were digested with 10 U Eco81I enzyme (Thermo Fisher Scientific) per reaction overnight at 37°C. The final products were electrophoresed on 3.5% agarose gel and visualised directly with ethidium bromide staining. Alleles were represented by 115bp and 38bp DNA fragments corresponding to the TGFB1[-509]*C allele and a 153bp DNA fragment corresponding to the TGFB1[-509]*T allele.

The genotyping of the TGFB2G[-875]A (rs3087465) polymorphism was performed by primer-introduced restriction analyses (PIRA-PCR) assay [13]. The primer sequences were the following: forward primer 5'-GCAAGAAAGGAAATTTGAAAGTTTGT-3' and reverse primer 5'-TCACCTGAATGCTTGTGCTTTT-3'. The PCR amplification was performed under the

Table I. Clinical characteristics of relapsing-remitting multiple sclerosis (RRMS) patients

Characteristics	RRMS patients N = 183
Age (mean \pm SD) in years	40.50 \pm 9.01
Gender, n (%)	
Female	132 (72.1%)
Male	51 (27.9%)
DMT, n (%)	
Interferon β	147 (80.3%)
Glatiramer acetate	26 (14.2%)
No treatment	10 (5.5%)
Mean duration of DMT (mean \pm SD) in months	57.45 \pm 37.19
Age of onset of the disease (mean \pm SD) in years	29.86 \pm 8.28
Onset of RRMS < 30 years, n (%) (mean \pm SD)	95 (51.9%) 23.21 \pm 3.83
Onset of RRMS \geq 30 years (mean \pm SD)	88 (48.1%) 37.05 \pm 5.19
Duration of the disease (mean \pm SD) in years	10.62 \pm 6.13
EDSS (mean \pm SD)	2.03 \pm 0.90

RRMS – relapsing-remitting multiple sclerosis, SD – standard deviation, DMT – disease-modifying treatment, EDSS – Expanded Disability Status Scale

following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 45 s at 94°C, for 45 s at 57°C and 45 s at 72°C, and completed with final extension cycle for 7 min at 72°C. The 124bp PCR products were digested with RsaI (10 U/μl) restriction enzyme at 37°C overnight. The final products were electrophoresed on 3.5% agarose gel and visualised directly with ethidium bromide staining. The TGFBR2[-875]*A allele produced two DNA fragments with lengths of 99bp and 25bp, while TGFBR2[-875]*G allele resulted in a fragment of 124bp.

All PCR reactions were performed in the GeneAmp PCR System 9700 (Applied Biosystems). The reagents for PCR and primers were provided by Thermo Fisher Scientific (USA) and Metabion GmbH (Germany).

Statistical analysis

Statistical data processing was performed using the Statistica software package (StatSoft Inc., USA) version 12.0. The differences in the distribution of genotype and allele frequencies between patients and controls were analysed using the χ^2 test. The Fisher exact test and Yates' corrected p -value (p_c) were used when the observed frequencies comprised a smaller group. The association between analysed genotypes of the *TGFB1* and *TGFB2* genes and risk of RRMS were estimated by calculating the odds ratios (ODs) and 95% confidence intervals (95% CI) using the StatPages.net website (<http://statpages.org/index.html>). The Hardy-Weinberg equilibrium was tested by comparing the observed genotype frequencies to the expected frequencies for cases and controls by the χ^2 test. All data were regarded as significant at $p \leq 0.05$.

Results

Association of TGFB1C[-509]T and TGFBR2G[-875]A polymorphisms with RRMS susceptibility

The different genotype and allele frequencies of the TGFB1C[-509]T and TGFBR2G[-875]A promoter polymorphisms in the cases and controls are presented in Table II. The observed genotype distribution for TGFB1C[-509]T and TGFBR2G[-875]A (rs1800469 and rs3087465) showed no deviation from Hardy-Weinberg equilibrium in the patients with RRMS ($\chi^2 = 0.026$, $p = 0.987$, $\chi^2 = 2.228$, $p = 0.328$) and controls ($\chi^2 = 0.977$, $p = 0.613$, $\chi^2 = 0.122$, $p = 0.940$,

respectively). The differences between cases and controls, in the distribution of the genotypes of both studied polymorphisms (rs1800469 and rs3087465), were non-significant (rs1800469: $\chi^2 = 2.302$, $p = 0.316$, rs3087465: $\chi^2 = 2.342$, $p = 0.31$). However, the analyses of data after stratification by sex showed that the frequency of TGFB1T[-509]T genotype and TGFB1[-509]*T-allele was decreased in RRMS men compared to healthy men (15.7% vs. 26.9%, 37.3% vs. 50.7%, respectively). Moreover, the homozygous TGFB1T[-509]T genotype was related to a significantly reduced risk of RRMS (OR = 0.360, 95% CI: 0.126-1.028, $p = 0.05$) referred to the TGFB1C[-509]C genotype among men. Also, TGFB1[-509]*T allele was more common in men with RRMS than among healthy men and was associated with a statistically significant protective effect (OR = 0.576, 95% CI: 0.341-0.974, $p = 0.039$). The observed distribution of genotypes of both polymorphisms was similar among groups of RRMS and healthy women.

The combined effect of the rs1800469 and rs3087465 polymorphisms on RRMS susceptibility

Further, we studied the effect of TGFB1C[-509]T and TGFBR2G[-875]A genotype combination for RRMS susceptibility in our groups. The results presented in Table III indicate that the combination of the TGFB1T[-509]T with TGFBR2G[-875]A genotypes as well as TGFB1T[-509]T and TGFBR2G[-875]A/TGFBR2A[-875]A are associated with significantly reduced risk of RRMS compared to TGFB1C[-509]C and TGFBR2G[-875]G genotypes (OR = 0.357, 95% CI: 0.136-0.938, $p = 0.032$; OR = 0.411, 95% CI: 0.173-0.976, $p = 0.040$). Similarly, when we combined the TGFB1T[-509]C/TGFB1T[-509]T and TGFBR2G[-875]A genotypes among men, compared to those carrying both wild-type homozygotes TGFB1C[-509]C and TGFBR2G[-875]G genotypes, the significance of protective effect increased (OR = 0.268, 95% CI: 0.088-0.818, $p = 0.018$). No significant association was observed between rs1800469 and rs3087465 among women with RRMS and controls.

Association of TGFB1C[-509]T and TGFBR2G[-875]A polymorphisms with the onset of RRMS disease

As regards to the importance of TGFB1C[-509]T and TGFBR2G[-875]A promoter polymorphisms for

Table II. Genotype and allele distribution of -509C/T TGFB1 and -875G/A TGFB2 polymorphisms in relapsing-remitting multiple sclerosis (RRMS) patients and healthy controls

Genotype	RRMS, n (%)	Controls, n (%)	OR (95% CI)	P-value
TGFB1 -509C/T (rs1800469)				
Total N	183	307		
CC	70 (38.3)	104 (33.9)	ref.	
CT	86 (47)	142 (46.2)	0.900 (0.601-1.348)	0.609
TT	27 (14.7)	61 (19.9)	0.658 (0.381-1.134)	0.131
CT/TT	113 (61.7)	203 (66.1)	0.827 (0.565-1.210)	0.328
C allele	226 (61.7)	350 (57)	ref.	
T allele	140 (38.3)	264 (43)	0.821 (0.630-1.070)	0.144
Female n	132	240		
CC	49 (37.1)	87 (36.3)	ref.	
CT	64 (48.5)	110 (45.8)	1.033 (0.648-1.647)	0.891
TT	19 (14.4)	43 (17.9)	0.785 (0.412-1.493)	0.459
CT/TT	83 (62.9)	153 (63.7)	0.963 (0.620-1.496)	0.867
C allele	162 (61.4)	284 (59.2)	ref.	
T allele	102 (38.6)	196 (40.8)	0.912 (0.671-1.241)	0.558
Male n	51	67		
CC	21 (41.2)	17 (25.4)	ref.	
CT	22 (43.1)	32 (47.7)	0.557 (0.241-1.288)	0.169
TT	8 (15.7)	18 (26.9)	0.360 (0.126-1.028)	0.053
CT/TT	30 (58.8)	50 (74.6)	0.486 (0.222-1.063)	0.069
CC vs. CT/TT			2.059 (0.941-4.507)	0.069
C allele	64 (62.7)	66 (49.3)	ref.	
T allele	38 (37.3)	68 (50.7)	0.576 (0.341-0.974)	0.039
TGFB2 -875G/A (rs3087465)				
Total N	183	307		
GG	125 (68.3)	193 (62.9)	ref.	
GA	49 (26.8)	102 (33.2)	0.742 (0.493-1.116)	0.151
AA	9 (4.9)	12 (3.9)	1.158 (0.474-2.829)	0.747
G allele	299 (81.7)	488(79.5)	ref.	
A allele	67 (18.3)	126 (20.5)	0.868 (0.624-1.207)	0.399
Female n	132	240		
GG	93 (70.4)	159 (66.3)	ref.	
GA	34 (25.8)	72 (30)	0.807 (0.449-1.307)	0.383
AA	5 (3.8)	9 (3.7)	0.950 (0.309-2.919)	0.928
G allele	220 (83.3)	390 (81.3)	ref.	
A allele	44 (16.7)	90 (18.7)	0.867 (0.583-1.289)	0.479
Male n	51	67		
GG	32 (62.8)	34 (50.7)	ref.	
GA	15 (29.4)	30 (44.8)	0.531 (0.242-1.165)	0.113
AA	4 (7.8)	3 (4.5)	1.417 (0.294-6.829)	0.663
G allele	79 (77.5)	98 (73.1)	ref.	
A allele	23 (22.5)	36 (26.9)	0.793 (0.434-1.446)	0.448

RRMS – relapsing-remitting multiple sclerosis, OR – odds ratios, CI – confidence intervals

Table III. Combination of genotypes -509C/T TGFB1 and -875G/A TGFB2 promoter polymorphisms

TGFB1 -509C/T	TGFB2 -875G/A	RRMS n (%)	Controls n (%)	OR (95%CI)	P-value
Total N		183	307		
CC	GG	47 (25.7)	70 (22.8)	ref.	
CC	GA	19 (10.4)	29 (9.5)	0.976 (0.491-1.939)	0.944
CC	AA	4 (2.2)	5 (1.6)	1.191 (0.304-4.669)	0.801
CC	GA/AA	23 (12.6)	34 (11.1)	1.008 (0.528-1.921)	0.982
CT	GG	59 (32.2)	91 (29.6)	0.966 (0.589-1.582)	0.890
CT	GA	24 (13.1)	48 (15.7)	0.745 (0.403-1.376)	0.346
CT	AA	3 (1.6)	3 (1)	1.489 (0.288-7.697)	0.633
CT	GA/AA	27 (14.7)	51 (16.6)	0.788 (0.435-1.430)	0.434
TT	GG	19 (10.4)	32 (10.4)	0.884 (0.449-1.741)	0.722
TT	GA	6 (3.3)	25 (8.1)	0.357 (0.136-0.938)	0.032
TT	AA	2 (1.1)	4 (1.3)	0.745 (0.131-4.231)	0.739
TT	GA+AA	8 (4.4)	29 (9.4)	0.411 (0.173-0.976)	0.040
CT/TT	GG	78 (42.6)	123 (40)	0.944 (0.593-1.505)	0.810
CT/TT	GA	30 (16.4)	73 (23.8)	0.612 (0.348-1.075)	0.087
CT/TT	GA/AA	35 (19.1)	80 (26.1)	0.652 (0.379-1.121)	0.121
Female n		132	240		
CC	GG	33 (25)	60 (25)	ref.	
CC	GA	13 (9.8)	23 (9.6)	1.028 (0.461-2.291)	0.947
CC	AA	3 (2.3)	4 (1.7)	1.364 (0.288-6.464)	0.695
CC	GA+AA	16 (12.1)	27 (11.3)	1.077 (0.509-2.281)	0.845
CT	GG	47 (35.6)	76 (31.7)	1.124 (0.643-1.967)	0.681
CT	GA	16 (12.1)	31 (12.9)	0.938 (0.449-1.963)	0.866
CT	AA	1 (0.8)	3 (1.2)	0.606 (0.061-6.061)	0.667
CT	GA+AA	17 (12.9)	34 (14.2)	0.909 (0.442-1.869)	0.795
TT	GG	13 (9.8)	23 (9.6)	1.028 (0.461-2.291)	0.947
TT	GA	5 (3.8)	18 (7.5)	0.505 (0.172-1.484)	0.209
TT	AA	1 (0.8)	2 (0.8)	0.909(0.079-10.407)	0.939
TT	GA/AA	6 (4.5)	20 (8.3)	0.545 (0.199-1.492)	0.233
Male n		51	67		
CC	GG	14 (27.4)	10 (14.9)	ref.	
CC	GA	6 (11.7)	6 (9)	0.714 (0.177-2.875)	0.729
CC	AA	1 (2)	1 (1.5)	0.714 (0.040-12.829)	0.819
CC	GA/AA	7 (13.7)	7 (10.4)	0.714 (0.190-2.688)	0.618
CT	GG	12 (23.5)	15 (22.4)	0.571 (0.188-1.736)	0.322
CT	GA	8 (15.7)	17 (25.4)	0.336 (0.105-1.081)	0.064
CT	GA/AA	10 (19.6)	17 (25.4)	0.420 (0.136-1.296)	0.128
TT	GG	6 (11.8)	9 (13.4)	0.467 (0.128-1.771)	0.265
TT	GA	1 (2)	7 (10.4)	0.102 (0.011-0.965)	0.066 ^c
TT	AA	1 (2)	2 (3)	0.357 (0.028-4.501)	0.411
TT	GA/AA	2 (4)	9 (13.4)	0.159 (0.028-0.899)	0.065 ^c
CT/TT	GA/AA	12 (6.6)	26 (8.5)	0.687 (0.316-1.496)	0.343
CT/TT	GA	9 (17.6)	24 (35.8)	0.268 (0.088-0.818)	0.018

RRMS – relapsing-remitting multiple sclerosis, OR – odds ratios, CI – confidence intervals, p^c – Yates’ corrected p value

clinical manifestation of RRMS, we found differences between genotype distribution among patients with early (under 30 years) and late (above 30 years) onset of the disease. Moreover, we investigated the combined effects of both polymorphism variants with the risk of the onset of the disease (Table IV). The frequency of the combined TGFB1C[-509]C and TGFB2G[-875]A genotype was overrepresented among patients with early onset of RRMS than patients with late onset (15.8% vs. 4.6%, OR = 3.913, 95% CI:

1.130-13.554, $p = 0.05$). Similarly, the patients with early onset showed a higher frequency of the combination variant of TGFB1C[-509]C and TGFB2G[-875]A/TGFB2A[-875]A than those with late onset of the disease (19% vs. 5.7%, OR = 3.757, 95% CI: 1.197-11.793, $p = 0.019$). Likewise, in women with an early onset of RRMS a higher frequency of combined effects of TGFB1C[-509]C and TGFB2G[-875]A genotypes as well as TGFB1C[-509]C and TGFB2G[-875]A/TGFB2A[-875]A genotypes were observed compared

Table IV. Association of -509C/T TGFB1 and -875G/A TGFB2 polymorphisms with onset of relapsing-remitting multiple sclerosis (RRMS)

TGFB1 -509C/T	TGFB2 -875G/A	Early onset n (%)	Late onset n (%)	OR (95% CI)	P-value
Total N		95	88		
CC	GG	23 (24.2)	24 (27.3)	ref.	
CC	GA	15 (15.8)	4 (4.6)	3.913 (1.130-13.554)	0.050 ^c
CC	AA	3 (3.2)	1 (1.1)	3.130 (0.303-32.314)	0.317
CC	GA/AA	18 (19)	5 (5.7)	3.757 (1.197-11.793)	0.019
CT	GG	25 (26.3)	34 (38.6)	0.767 (0.355-1.658)	0.500
CT	GA	15 (15.8)	9 (10.2)	1.739 (0.637-4.751)	0.278
CT	AA	1 (1)	2 (2.3)	0.522 (0.044-6.154)	0.600
CT	GA/AA	16 (16.8)	11 (12.5)	1.518 (0.583-3.953)	0.392
TT	GG	9 (9.5)	10 (11.4)	0.939 (0.323-2.729)	0.908
TT	GA	3 (3.2)	3 (3.4)	1.043 (0.191-5.709)	0.961
TT	AA	1 (1)	1 (1.1)	1.043 (0.062-17.686)	0.976
TT	GA/AA	4 (4.2)	4 (4.5)	1.043 (0.233-4.673)	0.956
CT/TT	GG	34 (35.8)	44 (50)	0.806 (0.390-1.667)	0.561
CT/TT	GA/AA	20 (21)	15 (17)	1.391 (0.577-3.356)	0.462
CC/TT	GA/AA	22 (23.2)	9 (10.2)	2.551 (0.973-6.686)	0.054
Female n		62	70		
CC	GG	11 (17.8)	19 (27.1)	ref.	
CC	GA	11 (17.8)	5 (7.1)	3.800 (1.044-13.830)	0.038
CC	AA	2 (3.2)	1 (1.4)	3.455 (0.280-42.622)	0.311
CC	AG/AA	13 (21)	6 (8.6)	3.742 (1.105-12.670)	0.030
CT	GG	19 (30.6)	27 (38.6)	1.215 (0.472-3.132)	0.686
CT	GA	8 (12.9)	9 (12.8)	1.535 (0.459-5.137)	0.485
CT	GA/AA	9 (14.5)	9 (12.8)	1.727 (0.528-5.651)	0.364
TT	GG	6 (9.7)	5 (7.1)	2.073 (0.511-8.405)	0.303
TT	GA	2 (3.2)	4 (5.7)	0.864 (0.135-5.507)	0.877
TT	GA/AA	4 (6.5)	4 (5.7)	1.727 (0.359-8.322)	0.493
Male n		33	18		
CC	GG	8 (24.2)	4 (22.2)	ref.	
CT	GG	7 (21.2)	6 (33.3)	0.583 (0.115-2.952)	0.513
CT	GA	5 (15.2)	3 (16.7)	0.833 (0.129-5.396)	0.848
CT	GA/AA	6 (18.2)	3 (16.7)	1.000 (0.160-6.255)	1.000
TT	GG	3 (9.1)	4 (22.2)	0.375 (0.055-2.555)	0.311

OR – odds ratios, CI – confidence intervals, p^c – Yates' corrected p value

with the women with the late onset (17.8% vs. 7.1%, OR = 3.800, 95% CI: 1.044-13.830, $p = 0.038$; 21% vs. 8.6%, OR = 3.742, 95% CI: 1.105-12.670, $p = 0.030$) (Table IV). Analyses in the group of men showed no significant association in the combination of genotypes with the age of onset.

Discussion

In this study, we examined the genetic association between the two promoter polymorphisms in genes encoded the regulatory TGF- β 1 cytokine and its receptor and RRMS. The association between TGFB1T[-509]T genotype and TGFB1[-509]*T allele was observed among males with RRMS, while the TGFB2G[-875]A polymorphism was not associated with RRMS. Moreover, when the patient's genotype contains at least one TGFB1[-509]*T allele (carriers of both TGFB1C[-509]T and TGFB1T[-509]T genotypes) in combination with TGFB2G[-875]A genotype, the genetic risk for RRMS was decreased approximately four-fold for men, compared to men carrying both wild-type homozygous TGFB1C[-509]C and TGFB2G[-875]G genotypes (OR = 0.268, $p = 0.018$).

The two investigated single-nucleotide polymorphisms (SNPs) are located at the promoter region of *TGFB1* and *TGFB2* genes, which encodes proteins involved in the TGF- β 1 signalling pathway. A lot of studies with the use of animal models have already revealed a crucial role for TGF- β 1 in differentiation and pathogenicity of autoreactive Th1 cells in MS development [4,16,19]. The immunosuppressive and tolerogenic role of TGF- β 1 points to its gene as a potent candidate for a genetic predisposition to multiple sclerosis. Unfortunately, the first linkage analysis of the two polymorphic markers (D19S223 and D19S224) located close to the *TGFB1* gene with MS has not yielded positive results [9]. McDonnell *et al.* demonstrated no association between two TGF- β 1 gene microsatellite markers and MS susceptibility among patients with relapsing-remitting and secondary progressive MS from Northern Ireland [20]. Green *et al.* investigated TGFB1 haplotypes, consisting of five closely linked single nucleotide polymorphisms within the *TGFB1* locus: two occurring in the promoter region G[-800]A, C[-509]T, two in exon 1 (Leu10Pro)/rs1982073, (Arg25Pro)/rs1800471, and one in exon 5 (Thr263Ile)/rs1800474, by linkage analysis, and reported no significant association with MS. However, testing of individual *TGFB1* gene polymorphisms revealed significant associations between patients with "mild"

MS (EDSS < 3) and wild-type genotypes of both *TGFB1*C[-509]T and Leu10Pro SNPs. The authors concluded that the *TGFB1* gene variability influences disease expression rather than disease susceptibility [6]. Similar results were observed by Weinshenker *et al.* [35]. Among MS patients, the TGFB1[-509]*C allele was associated with higher disease severity. Those studies did not provide clear answers about the role of *TGFB1* gene polymorphisms in MS development. Moreover, several polymorphisms in the *TGFB1* gene affecting TGF- β 1 production have been identified [5,18].

In general, our results for the association between the promoter TGFB1C[-509]T polymorphism and RRMS susceptibility are in line with all the studies cited above. However, when we stratified the patients' group by gender a significant difference was observed between the males with TGFB1T[-509]T genotype in cases and controls. Our results demonstrated that TGFB1[-509]*T allele and TGFB1T[-509]T genotype may protect against RRMS in men. Similar results for the gender dependency have been published for two *TGFB1* gene SNPs located in the first exon at +869 position (Leu10Pro) and +915 position (Arg25Pro) associated with MS in Dutch Caucasian patients. The authors concluded that TGFB1T[+869]C (Leu10Pro) (rs1982073) gene polymorphism is associated with MS susceptibility, especially in males [27]. The sex-dependent differences in gene expression, particularly for the *TGFB1* gene, could explain these results. In our recent study, we demonstrated that TGF- β 1 serum levels depend on the TGFB1C[-509]T genotype in combination with gender and age. The highest levels of the cytokine occur in males with homozygous TGFB1T[-509]T genotype [33]. Taking into account that men with TT genotype produced higher quantities of TGF- β 1 in serum, this genotype could be a possible protective factor for MS development. This is in line with the observation of the neuroprotective effects of TGF- β 1 [3].

Recently, the role that TGF- β 1 plays in the generation and regulation of encephalitogenic T cells and the function of CD4 regulatory T cells in multiple sclerosis has been elucidated [16,17]. The inability of Tregs in patients with MS to suppress autoimmunity could be attributed to decreased TGFB2 expression on the T-cell surface. This effect can be mediated by promoter polymorphism causing a G-to-A transition at position -875 in the *TGFB2* gene. Moreover, Seijo *et al.* reported that the presence of polymorphic TGF-

BR2[-875]*A in the promoter region enhanced the transcription activity of TGFB2 in normal epithelial cells [28]. We did not find a significant association between genotypes of this polymorphism and MS susceptibility, although the patients with heterozygous genotype showed a trend towards diminished frequency in the RRMS group. The most significant association was observed when the genotypes of the two polymorphisms were combined. Obviously, the combination of TGFB1T[-509]T/TGFB2G[-875]A genotypes has the most protective effect. In our studied group of RRMS patients, male carriers of the two genotypes displayed four-fold lower susceptibility than non-carriers (OR = 0.268, $p = 0.018$). At the same time, the combination of the TGFB1C[-509]C genotype with TGFB2G[-875]A/TGFB2A[-875]A was a much more susceptible genotype for early onset of the disease (OR = 3.757, $p = 0.019$). The results clearly suggest the significance of both polymorphisms for MS susceptibility. Thus, our findings confirm previous results from many other studies on the role of the TGF-β1 signalling pathway in the development of this autoimmune disease mediated by differentiation of T-cells. Recently, new evidence for the impact of miRNA on the TGF-β signalling pathway and its dysregulation in multiple sclerosis has been found [29].

Nowadays, there is a consensus that the enhancement of TGF-β1 signalling could positively modulate Tregs cell differentiation and diminishes the activities of encephalopathic Th-17 cells in MS patients. Moreover, TGF-β1 can directly enhance IL-10 production, which negatively regulates the function of effector myelin-specific Th1 cells in MS patients [11].

We should mention some limitations of our study. The patient group consisted only of subjects with RRMS. Patients with PPMS or SPMS were not included. The number of male patients with RRMS was relatively small; therefore, our results are preliminary and need confirmation with the use of a larger cohort of patients. MS is a complex disease; thus, other genetic and environmental factors certainly also affect disease development.

In summary, for the first time, we demonstrate a link between the TGFB1C[-509]T genotype and MS. Male carriers of at least one TGFB1[-509]*T allele have a lower genetic risk of MS development, which is additionally reduced when the genotype coexists with the TGFB2[-875]*A allele. This combined genotype played a protective role for all patients in

our group, while the combined genotype including TGFB1C[-509]C genotype instead of TGFB1T[-509]T seems to predispose to early onset of MS development.

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Disclosure

The authors report no conflict of interest.

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