ORIGINAL ARTICLE

Correlations of TNF- α gene promoter polymorphisms with the risk of thymoma-associated myasthenia gravis in a northern Chinese Han population

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This study was performed with the aim to investigate the correlations of tumor necrosis factor- α (TNF- α) gene promoter polymorphisms with the risk of thymoma-associated myasthenia gravis (T-MG) in a northern Chinese Han population. Between June 2005 and June 2015, 305 MG patients (150 males and 155 females, MG group) and 293 healthy volunteers (negative control (NC) group) were enrolled in this study. Among the MG patients, there were 121 patients with thymoma-associated MG (T-MG group) and 184 without T-MG (NT-MG group). Enzyme-linked immunosorbent assay (ELISA) was used for the serum TNF- α level. Polymerase chain reaction–restriction fragment length polymorphism was conducted to determine genotype and allele frequencies of TNF- α gene promoter -1031T/C, -857C/T and -863C/A. The haplotype was analyzed with the SHEsis software. Logistic regression analysis was performed for correlations between TNF- α gene promoter polymorphisms and the risk of T-MG. The T-MG group had higher frequencies of the CT/TT genotype and T allele of -857C/T than the NT-MG and NC groups. The frequencies of the CC genotype and C allele of -1031T/C were higher in the T-MG group than in the NT-MG and NC groups, and higher in male patients in the T-MG group than in male patients in the NC group. TTA and TTC haplotypes exhibited lower frequencies in the T-MG group than in the NT-MG group. The ocular MG patients exhibited lower frequencies of the TT genotype and T allele of -857C/T than the generalized MG patients did. The TNF- α level was elevated in the T-MG group compared with that in the NC and NT-MG groups, indicating that the TC+CC and CT+TT genotypes were increased compared with the TT and CC genotypes in the -1031T/C and -857C/T, respectively. Logistic regression analysis suggested that expressions of anti-acetylcholine receptor antibodies, Osserman's classification, -1031T/C and -857C/T polymorphisms and the TTA haplotype were the independent risk factors for T-MG. These findings reveal that TNF- α -1031T/C and -857C/T polymorphisms and the TTA haplotype may be correlated with the occurrence of T-MG in a Northern Chinese Han population.

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INTRODUCTION

Myasthenia Gravis (MG) is known for many years as an autoimmune disorder in the neuromuscular junction, typically characterized by weakness of skeletal muscles.¹ It is a relatively rare disease with an estimated prevalence ranging from 131 to 145 cases in 1 million, which has been increasing in the last two decades.² Thymoma is a thymic epithelial tumor rare in young people below 20 years of age, and thymoma in adults is found to be commonly related with varied autoimmune diseases, the most frequently with MG.³ Thymoma-associated MG (T-MG) is considered to be more aggressive compared with nonthymoma-associated MG (NT-MG), and its prognosis mainly depends on the effects of long-term immunosuppressive therapy, so that thymoma is demonstrated as a negative prognostic factor for MG patients.⁴ In most cases, MG is believed to be caused by pathogenic auto-antibodies targeting the skeletal muscle acetylcholine receptor (AChR), and, in others, non-AChR components in postsynaptic muscle endplate may also function as the target for autoimmune attack.⁵ Therefore, other factors for MG susceptibility should be of great significance

to be found, and tumor necrosis factor- α (TNF- α) may be involved.

TNF- α is recognized as a potent pro-inflammatory cytokine; its synthesis and secretion are implicated in multiple pathologies.⁶ It has been evidenced to act as a critical role in the MG pathogenesis, and TNF- α -blocking agents have shown significant clinical efficacy and are proposed for MG treatment.⁷ TNF- α gene promoter polymorphisms have been demonstrated to be closely linked with disease pathology. To list a few, TNF- α -1031T/C and -863C/A polymorphisms are closely associated with the risk, clinical manifestation and progression of thyroid-associated ophthalmopathy (TAO).⁸ In addition, TNF- α -857C/T polymorphisms have been demonstrated to be an independent risk factor in the pathogenesis of gastric cancer.⁹ It has also been demonstrated early that the polymorphisms in the TNF region are associated with MG combined with thymic hyperplasia.¹⁰ Therefore, we hypothesized that there was an intensive association between polymorphisms in the TNF- α promoter region and the susceptibility of T-MG. However, there are few studies on the relationship between the TNF- α promoter polymorphisms and the progress of

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Table 1. Primer sequences of TNF- α -1031T/C, -863C/A and -857C/T

SNP	Primer sequence	Length (bp)	Allele
-1031 T/C	F: 5'-GTGGGGAGAACAAAAGGATAAGG-3' R: 5'-GACAGCCCCGGGGAGTGAAT-3'	552	T/C
-857C/T	F: 5'-GCGGCTCTGAGGAATGGGTTAC-3' R: 5'-TTGTCAGGGGATGTGGCGTCTG-3'	129	C/T
-863C/A	F: 5'-GGCTCTGAGGAATGGGTTAC-3' R: 5'-CTACATGGCCTGTCTTCGTTACG-3'	126	C/A

Abbreviations: F, forward; R, reverse; SNP, single-nucleotide polymorphism; TNF- α , tumor necrosis factor- α .

T-MG. On the basis of the situation, our study aims to explore the correlations of TNF- α gene promoter polymorphisms with the risk of T-MG in a Northern Chinese Han population.

MATERIALS AND METHODS

Study subjects

From June 2005 to June 2015, 305 MG outpatients in the Department of Neurology of Tianjin Medical University General Hospital were recruited as the MG group, among which there were 150 males and 155 females with a mean age of 36.3 ± 15.2 years. All included patients fulfilled the following diagnostic criteria for MG:¹¹ (1) the affected skeletal muscle is easily fatigued, and the symptoms exhibit fluctuation and improve after rest; (2) fatigue test is positive; (3) neostigmine test is positive; (4) the amplitude of muscle action potential decreases at least 10% in repetitive electrical stimulation; and (5) acetylcholine receptor (AChR) antibody is positive. The inclusion criteria were as follows: patients met the diagnostic criteria for MG and belong to a Northern Chinese Han population. The exclusion criteria were as follows: (1) patients without complete clinical data, family disease history or previous history; (2) female patients in pregnancy or lactation; (3) patients diagnosed with other autoimmune diseases or inflammatory diseases; and (4) patients diagnosed with the heart and lungs diseases. Simultaneously, 293 healthy volunteers (128 males and 165 females, with the mean age of 34.8 ± 8.3 years) having physical examination were selected as the negative control (NC) group. These healthy controls were Northern Chinese Han populations, without autoimmune diseases or family disease history. There were no statistically significant differences in the age and gender between the MG and NC groups (both $P > 0.05$), which suggested that the MG and NC groups were comparable. The protocol got the approval of the Ethics Committee of Tianjin Medical University General Hospital, and informed consent in written form was obtained from all subjects.

The diagnostic criteria of T-MG are as follows:¹² fatigue test is positive, the titer of serum AChR antibody (anti-AChR) increases and anticholinesterase agents' test is positive; and X-ray chest image or computed tomography and magnetic resonance imaging of the chest indicate mass in anterior mediastinum. The MG patients were further assigned into the T-MG group (patients with thymoma-associated MG; $n = 121$) and the NT-MG group (patients without thymoma-associated MG; $n = 184$). Of the 305 MG patients, there were 110 cases with positive anti-AChR and 195 cases with negative anti-AChR. Besides, according to Osserman's classification,¹³ there were 126 ocular MG patients and 179 generalized MG patients.

Extraction of genomic DNA

Venous blood (5 ml) was taken from every fasting subject under sterile conditions and placed into 2% ethylene diamine tetraacetic acid anticoagulated eppendorf tube. Genomic DNA was extracted in accordance with the kit instructions (Promega, Madison, WI, USA) and then reserved at -20°C .

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used for the TNF- α level in the serum according to the ELISA kit (Jinmei Company, Shenzhen, Guangdong, China). Peripheral venous blood (5 ml) was extracted from all subjects on their empty stomach, among which 2 ml was added with ethylene diamine tetraacetic acid and reserved at -80°C for DNA extraction. The rest 3 ml of blood was put still at the room temperature

for 30 min, and centrifuged (2000 r min^{-1}) for 15 min with serum collected. The 96-well plate coating anti-human TNF- α was added with samples and standards of various concentrations, following incubation for 60 min at 37°C . The biotinylated anti-human TNF- α was added again after the plate was washed, and incubated for 30 min at 37°C , treated with enzyme conjugate for 30 min at 37°C and developed. The optical density value was recorded at the wavelength of 450 nm. The standard curve was for result estimation with TNF- α concentration presented as pg ml^{-1} .

Polymerase chain reaction–restriction fragment length polymorphism

TNF- α gene sequence was retrieved from Gen Bank. Using the DNA-star software (Lynnon BioSoft, Vaudreuil, Canada), the primers of TNF- α gene promoter -1031T/C, -857C/T and -863C/A (Table 1) were designed by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China), the specificity of which was detected by the Blast software of NCBI. Owing to the absence of natural restriction site in -863C/A, polymerase chain reaction (PCR) method with a mismatched base was used, that is, a mismatched base (A $\rightarrow$ C) was inserted into the second of the 3' end of its reverse primer. After being synthesized, the primers were added with double distilled water to reach $50 \text{ pmol } \mu\text{l}^{-1}$ and stored at -20°C . For PCR–restriction fragment length polymorphism, specific restriction enzymes were used to identify the specific sites of the PCR products, and then the PCR products were detected by gel electrophoresis; next, the genotypes were detected according to the size of the product fragments.

PCR amplification

The reaction system of TNF- α -1031 T/C and -863C/A was $30 \text{ } \mu\text{l}$ in total, containing $4 \text{ } \mu\text{l}$ of DNA template, $3 \text{ } \mu\text{l}$ of $10 \times \text{dNTP}$, $3 \text{ } \mu\text{l}$ of $10 \times \text{Buffer}$, $0.5 \text{ } \mu\text{l}$ of $10 \text{ } \mu\text{mol l}^{-1}$ forward primer, $0.5 \text{ } \mu\text{l}$ of $10 \text{ } \mu\text{mol l}^{-1}$ reverse primer, $1.5 \text{ } \mu\text{l}$ of $25 \text{ mmol l}^{-1} \text{ MgCl}_2$ and $2 \text{ } \mu\text{l}$ of $1 \text{ U } \mu\text{l}^{-1}$ Taq DNA. Reaction condition for -1031 T/C was as follows: predenaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 1 min and finally extension at 72°C for 10 min. Reaction condition for -863C/A was as follows: predenaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s and finally extension at 72°C for 10 min. The reaction system of TNF- α -857C/T was $50 \text{ } \mu\text{l}$, consisting of $6 \text{ } \mu\text{l}$ of DNA template, $5 \text{ } \mu\text{l}$ of $10 \times \text{Buffer}$ with KCl, $1 \text{ } \mu\text{l}$ of $10 \text{ } \mu\text{mol l}^{-1}$ forward primer, $1 \text{ } \mu\text{l}$ of $10 \text{ } \mu\text{mol l}^{-1}$ reverse primer, $1 \text{ } \mu\text{l}$ of $10 \text{ mmol l}^{-1} \text{ dNTP}$, $5 \text{ } \mu\text{l}$ of $25 \text{ mmol l}^{-1} \text{ MgCl}_2$ and $2 \text{ } \mu\text{l}$ of $1 \text{ U } \mu\text{l}^{-1}$ Taq DNA (Fermentas, Hanover, MD, USA). Reaction condition for -857C/T was as follows: predenaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 1 min and finally extension at 72°C for 10 min. The PCR product was subjected to 8% polyacrylamide gel electrophoresis.

Restriction enzyme digestion

The reaction system of TNF- α -1031 T/C and -857C/T was $10 \text{ } \mu\text{l}$, containing $5 \text{ } \mu\text{l}$ of PCR product, $1 \text{ } \mu\text{l}$ of $10 \times \text{Buffer}$, $0.1 \text{ } \mu\text{l}$ of Bqil and deionized water. After mixing, the reaction system was subjected to a hybrid box or water bath at 37°C overnight. The reaction system of TNF- α -863C/A was $10 \text{ } \mu\text{l}$, consisting of $4 \text{ } \mu\text{l}$ of PCR product, $1 \text{ } \mu\text{l}$ of $10 \times \text{Buffer}$, $0.08 \text{ } \mu\text{l}$ of Bqil and deionized water. After mixing, the reaction system was subjected to a water bath at 65°C for 4 h and then cooled to 4°C . Subsequently, the product of enzyme digestion ($10 \text{ } \mu\text{l}$) was mixed with $6 \times \text{Buffer}$ at a ratio of 10:1. Agarose gel electrophoresis (2.5%, containing $0.5 \text{ } \mu\text{g ml}^{-1}$ ethidium

Table 2. Comparison of baseline characteristics among the NC, T-MG and NT-MG groups

Baseline characteristic	NC group (n = 293)	NT-MG group (N = 184)	T-MG group (N = 121)	P ¹	P ²	P ³
Gender (male/female)	128/165	88/96	62/59	0.377	0.161	0.560
Mean age (year)	34.8 $\pm$ 8.3	35.5 $\pm$ 13.8	36.8 $\pm$ 16.1	0.489	0.097	0.452
Smoking (yes/no)	139/154	99/85	60/61	0.176	0.572	0.471
Drinking (yes/no)	134/159	99/85	66/55	0.498	0.103	0.336
Anti-AchR				0.531	0.018	0.099
Positive	86 (29.4)	59 (32.1)	51 (42.1)			
Negative	207 (70.6)	125 (67.9)	70 (57.9)			
Osseman's classification				—	—	0.004
Ocular		88 (47.8)	38 (31.4)			
Generalized		96 (52.2)	83 (68.6)			
Thymoma histology				—	—	< 0.001
Thymoma		21 (11.4)	72 (59.5)			
A		5 (2.7)	3 (2.5)			
AB		3 (1.6)	7 (5.8)			
B1		6 (3.3)	31 (25.6)			
B2		5 (2.7)	29 (23.9)			
B3		2 (1.1)	2 (1.7)			
Hyperplasia		59 (32.1)	24 (19.8)			
Atrophy of thymus gland		54 (29.3)	11 (9.1)			
Normal thymus gland		50 (27.2)	14 (11.6)			

Abbreviations: anti-AchR, acetylcholine receptor antibody; NC, negative control; NT-MG, nonthymoma-associated myasthenia gravis; P¹, comparison between the NC and NT-MG groups; P², comparison between the NC and T-MG groups; P³, comparison between the NT-MG and T-MG groups; T-MG, thymoma-associated myasthenia gravis.

Figure 1. Sequencing results of PCR products of TNF- α -1031T/C, -863C/A and -857C/T. Note: (a) PCR products of TNF- α -1031T/C; (b) PCR products of TNF- α -863C/A; (c) PCR products of TNF- α -857C/T. TNF- α , tumor necrosis factor- α ; PCR, polymerase chain reaction.

bromide) was performed for 40 min in 0.5 $\times$ 1, 1, 2, 2-tetrabromoethane Buffer at 80 V and observed using the Ultraviolet Imaging System (Uvidoc, Uvitec Cambridge, MA, USA).

Statistical analysis

Statistical package for the social sciences (SPSS) software 19.0 (SPSS, Chicago, IL, USA) was applied for data analysis. Hardy-Weinberg equilibrium (HWE) was performed to determine whether the subjects had group representation. The χ^2 -test was conducted to analyze whether the subjects in the MG and NC groups were in a state of equilibrium, and $P \geq 0.05$ presented good group representation. Measurement data were expressed by mean $\pm$ s.d. deviation and compared using *t*-test. Categorical data were shown as a ratio or percentage and compared using χ^2 -test. $P < 0.05$ was considered significantly different. Logistic regression analysis was performed to calculate the odds ratio and 95% confidence interval, which were used to analyze the independent risk factors for T-MG.

RESULTS

Comparison of baseline characteristics among the NC, T-MG and NT-MG groups

There were 293 individuals in the NC group, including 128 males and 165 females, with a mean age of 34.8 ± 8.3 years. There were 184 patients in the NT-MG group, including 88 males and 96 females, with a mean age of 35.5 ± 13.8 years. The T-MG group was composed of 121 subjects, including 62 males and 59 females, with a mean age of 36.8 ± 16.1 years. There were no statistically significant differences in gender, mean age, smoking or drinking among the NC, T-MG and NT-MG groups (all $P > 0.05$). The positive rate of anti-AchR in the T-MG group was higher than that in the NC group ($P < 0.05$). The ratio of generalized MG patients in the T-MG group was higher than that in the NT-MG group ($P < 0.05$). The majority of patients in the T-MG group had B1 or B2 thymoma, and the majority of patients in the NT-MG

Table 3. Comparison of genotype and allele frequencies of TNF- α -1031T/C, -863C/A and -857C/T among the NC, T-MG and NT-MG groups

SNP	Genotype/allele	NC group	NT-MG group	T-MG group	P ¹	OR (95% CI)	P ²	OR (95% CI)	P ³	OR (95% CI)
-1031T/C	TT	194 (66.21)	124 (67.39)	69 (57.02)	0.76	0.939 (0.626–1.409)	Ref.	1.218 (0.765–1.941)	0.312	0.771 (0.464–1.278)
	TC	90 (30.72)	54 (29.35)	39 (32.23)	0.938	1.043 (0.362–3.003)	0.405	4.061 (1.662–9.923)	0.005	3.894 (1.416–10.710)
	CC	9 (3.07)	6 (3.26)	13 (10.74)	0.896	0.981 (0.731–1.315)	0.71	1.069 (0.751–1.522)	<0.001	2.962 (2.027–4.328)
	TC+TT	284 (96.93)	178 (96.74)	108 (89.26)	0.847	0.967 (0.690–1.357)	Ref.	1.625 (1.142–2.313)	0.009	0.595 (0.403–0.879)
	T	478 (81.57)	302 (82.07)	177 (73.14)	0.615	0.905 (0.615–1.334)	Ref.	1.605 (1.005–2.563)	0.026	0.564 (0.340–0.936)
-857C/T	C	108 (18.43)	66 (17.93)	65 (26.86)	0.482	1.335 (0.594–3.000)	<0.001	6.838 (3.294–14.190)	<0.001	0.195 (0.090–0.422)
	CC	162 (55.29)	102 (55.43)	44 (36.36)	0.792	0.951 (0.656–1.379)	0.001	2.164 (1.399–3.348)	0.001	0.440 (0.274–0.704)
	CT	117 (39.93)	70 (38.04)	51 (42.15)	0.929	1.014 (0.750–1.371)	<0.001	2.254 (1.642–3.093)	<0.001	0.450 (0.318–0.637)
	TT	14 (4.78)	12 (6.52)	26 (21.49)	0.64	0.906 (0.600–1.369)	Ref.	0.984 (0.617–1.569)	0.751	0.921 (0.552–1.535)
	CT+TT	131 (44.71)	82 (44.57)	77 (63.64)	0.343	1.410 (0.691–2.875)	0.87	1.076 (0.449–2.581)	0.554	1.310 (0.534–3.213)
-863C/A	C	441 (75.26)	274 (74.46)	139 (57.44)	0.957	0.987 (0.676–1.450)	Ref.	1.000 (0.645–1.549)	0.967	0.990 (0.616–1.592)
	CC	145 (24.74)	94 (25.54)	103 (42.56)	0.676	1.069 (0.782–1.462)	0.942	1.013 (0.705–1.456)	0.789	1.055 (0.714–1.558)
	CA	184 (62.80)	116 (63.04)	76 (62.81)						
	CA	91 (31.06)	52 (28.26)	37 (30.58)						
	AA	18 (6.14)	16 (8.70)	8 (6.61)						
	CA+AA	109 (37.20)	68 (36.96)	45 (37.19)						
	C	459 (78.33)	284 (77.17)	189 (78.10)						
	A	127 (21.67)	84 (22.83)	53 (78.10)						

Abbreviations: CI, confidence interval; NC, negative control; NT-MG, nontymoma-associated myasthenia gravis; OR, odd ratio; P¹, comparison between the NC and NT-MG groups; P², comparison between the NC and T-MG groups; P³, comparison between the NT-MG and T-MG groups; SNP, single-nucleotide polymorphism; T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor- α .

group had hyperplasia, atrophy of thymus gland or normal thymus gland (all $P < 0.05$; Table 2).

PCR products of TNF- α -1031T/C, -857C/T and -863C/A

Following digestion by BqII, -1031T/C showed the wild-type TT (293 and 259 bp), the heterozygous mutant type TC (293, 259, 180 and 113 bp) and the homozygous mutant type CC (259, 180 and 113 bp; Figure 1a). The -857C/T showed the wild-type CC (110 and 19 bp), the heterozygous mutant type CT (129, 110 and 19 bp) and the homozygous mutant type TT (129 bp; Figure 1b). The -863C/A showed the wild-type CC (126 bp), the heterozygous mutant type TC (126, 105 and 21 bp) and the homozygous mutant type AA (105 and 21 bp; Figure 1c).

Genotype and allele frequencies of TNF- α -1031 T/C, -863C/A and -857C/T among the NC, T-MG and NT-MG groups

The genotype distribution of TNF- α -1031 T/C, -863C/A and -857C/T among the NC, T-MG and NT-MG groups was subjected to HWE, and the results indicated that -1031 T/C, -863C/A and -857C/T were consistent with HWE (all $P > 0.05$), which indicated that the frequencies of those genotypes were in a state of equilibrium, with group representation. As shown in Table 3, the frequencies of the CC genotype and the C allele of -1031 T/C were higher in the T-MG group than those in the NT-MG and NC groups, and the frequencies of the CT/TT genotype and the T allele of -857C/T were higher in the T-MG group than those in the NT-MG and NC groups (all $P < 0.05$). However, no significant differences were found in the genotype and allele frequency of -863C/A among the NC, T-MG and NT-MG groups (all $P > 0.05$).

Association of genotype and allele frequencies of TNF- α -1031 T/C, -863C/A and -857C/T with the clinicopathological characteristics for MG patients

The frequencies of the CC genotype and the C allele of -1031 T/C were higher in the T-MG group than in the NT-MG and NC groups (both $P < 0.05$), and higher in male patients in the T-MG group than in male patients in the NC group ($P < 0.05$; Table 4). The frequencies of the TT genotype and the T allele of -857C/T were higher in the T-MG group than in the NT-MG and NC groups (both $P < 0.05$). The ocular MG patients exhibited lower frequencies of the TT genotype and the T allele of -857C/T than the generalized MG patients (both $P < 0.05$; Table 5). However, no significant difference was found in the genotype and allele frequency of -863C/A in terms of the presence of T-MG, gender, Osserman's classification and clinical stage (all $P > 0.05$; Table 6).

Haplotype analysis of TNF- α -1031 T/C, -863C/A and -857C/T among the NC, T-MG and NT-MG groups

Using the SHEsis software (<http://analysis.bio-x.cn/SHEsisMain.htm>), haplotypes of TNF- α -1031 T/C, -863C/A and -857C/T in the T-MG and NT-MG groups were analyzed. The haplotype of frequency less than 3% was ignored. As shown in Table 7, TTA and TTC haplotypes exhibited lower frequencies in the T-MG group than in the NT-MG group (both $P < 0.05$). However, no significant difference was observed in other haplotypes between the T-MG and NT-MG groups (all $P > 0.05$).

Serum TNF- α level detected by ELISA among the NC, T-MG and NT-MG groups

ELISA showed that TNF- α level was elevated in the T-MG and NT-MG groups compared with that in the NC group, and TNF- α level was significantly increased in the T-MG group as compared to that in the NT-MG group ($P < 0.05$; Figure 2). TNF- α level in the serum was analyzed with various genotypes, indicating that the levels in the TC+CC and CT+TT genotypes were increased

Table 4. Genotype and allele frequencies of TNF- α -1031 T/C between the NT-MG and T-MG groups

Subject	Case	Genotype			Allele	
		TT	TC	CC	T	C
NC group	293	194 (66.21)	90 (30.72)	9 (3.07)	478 (81.57)	108 (18.43)
Male	128	87 (67.97)	40 (31.25)	1 (0.78)	214 (83.59)	42 (16.41)
Female	165	107 (64.85)	50 (30.30)	8 (4.85)	264 (80.00)	66 (20.00)
Anti-AchR-positive	86	56 (65.12)	26 (30.23)	4 (4.65)	138 (80.23)	34 (19.77)
Anti-AchR-negative	207	138 (66.67)	64 (30.92)	5 (2.42)	340 (82.13)	74 (17.87)
NT-MG group	184	124 (67.39)	54 (29.35)	6 (3.26)	302 (82.07)	66 (17.93)
T-MG group	121	69 (57.02)	39 (32.23)	13 (10.74) ^{a,b}	177 (73.14)	65 (26.86) ^{c,d}
Male	150	94 (62.67)	42 (28.00)	14 (9.33) ^e	230 (76.67)	70 (23.33) ^f
Female	155	99 (63.87)	51 (32.90)	5 (3.23)	249 (80.32)	61 (19.68)
Anti-AchR-positive	110	65 (59.09)	40 (36.36)	5 (4.55)	170 (77.27)	50 (22.72)
Anti-AchR-negative	195	128 (65.64)	53 (27.18)	14 (7.18)	309 (79.23)	81 (20.77)
Ocular	126	79 (62.70)	43 (34.13)	4 (3.17)	201 (79.76)	51 (20.24)
Generalized	179	114 (63.69)	50 (27.93)	15 (8.38)	278 (77.65)	80 (22.35)

Abbreviations: anti-AchR, acetylcholine receptor antibody; NT-MG, nonthymoma-associated myasthenia gravis; T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor- α . ^a $P < 0.05$ compared with the frequency of the CC genotype in the NT-MG group. ^b $P < 0.05$ compared with the frequency of the CC genotype in the NC group. ^c $P < 0.05$ compared with the frequency of the C allele in the NT-MG group. ^d $P < 0.05$ compared with the frequency of the C allele in the NC group. ^e $P < 0.05$ compared with the frequency of the CC genotype in the male NC patients. ^f $P < 0.05$ compared with the frequency of the C allele in the male NC patients.

Table 5. Genotype and allele frequencies of TNF- α -857C/T between the T-MG and NT-MG groups

Subject	Case	Genotype			Allele	
		CC	CT	TT	C	T
NC group	293	162 (55.29)	117 (39.93)	14 (4.78)	441 (75.26)	145 (24.74)
Male	128	70 (54.69)	51 (39.84)	7 (5.47)	191 (74.61)	65 (25.39)
Female	165	92 (55.76)	66 (40)	7 (4.24)	250 (75.76)	80 (24.24)
Anti-AchR-positive	86	47 (54.65)	35 (40.70)	4 (4.65)	129 (75.00)	43 (25.00)
Anti-AchR-negative	207	115 (55.56)	82 (39.61)	10 (4.83)	312 (75.36)	102 (24.64)
NT-MG group	184	102 (55.43)	70 (38.04)	12 (6.52)	274 (82.07)	94 (17.93)
T-MG group	121	44 (36.36)	51 (42.15)	26 (21.49) ^{a,b}	139 (57.44)	103 (42.56) ^{c,d}
Male	150	72 (48.00)	59 (39.33)	19 (12.67)	203 (67.67)	97 (32.33)
Female	155	74 (47.74)	62 (40.00)	19 (12.26)	210 (67.74)	100 (32.26)
Anti-AchR-positive	110	54 (49.09)	47 (42.73)	9 (8.18)	155 (70.45)	65 (29.55)
Anti-AchR-negative	195	92 (47.18)	74 (37.95)	29 (14.87)	258 (66.15)	132 (33.85)
Ocular	126	77 (61.11)	35 (27.78)	14 (11.11)	189 (75.00)	63 (25.00)
Generalized	179	69 (38.55)	86 (48.04)	24 (13.41) ^e	224 (62.57)	134 (37.43) ^f

Abbreviations: anti-AchR, acetylcholine receptor antibody; NT-MG, nonthymoma-associated myasthenia gravis; T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor- α . ^a $P < 0.05$ compared with the frequency of the TT genotype in the NT-MG group. ^b $P < 0.05$ compared with the frequency of the TT genotype in the NC group. ^c $P < 0.05$ compared with the frequency of the T allele in the NT-MG group. ^d $P < 0.05$ compared with the frequency of the T allele in the NC group. ^e $P < 0.05$ compared with the frequency of the TT genotype in patients with ocular MG. ^f $P < 0.05$ compared with the frequency of the T allele in patients with ocular MG.

compared with those in the TT and CC in the -1031 T/C and -857C/T, respectively ($P < 0.05$), while there was no significant difference among the CC and CA+AA genotype in the 863C/A, as presented in Table 8 ($P > 0.05$).

Logistic regression analysis of the independent risk factors for T-MG

With the presence of T-MG as a dependent variable and with age, gender, -1031 T/C, -857C/T, anti-AchR, Osserman's classification and the TTA and TTC haplotypes as independent variables, multivariate logistic regression analysis was performed. The results implied that anti-AchR, Osserman's classification, -1031 T/C and

-857C/T polymorphisms and the TTA haplotype were the independent risk factors of T-MG (all $P < 0.05$, Table 9).

DISCUSSION

MG is a well-documented chronic autoimmune disease resulting from an antibody-associated autoimmune attack targeting AchR at the neuromuscular junctions, with obvious clinical features including muscular weakness and fatigability in skeletal muscles.¹⁴ The relationship between thymoma and MG was first identified by Weigert in 1901, and thymoma is pointed out to be a negative prognostic factor of the outcome for MG.¹⁵ A study further reports that T-MG occurs in ~80–85% of MG patients with

Table 6. Genotype and allele frequencies of TNF- α -863C/A between the T-MG and NT-MG groups

Subject	Case	Genotype			Allele	
		CC	CA	AA	C	A
NC group	293	184 (62.80)	91 (31.06)	18 (6.14)	459 (78.33)	127 (21.67)
Male	128	75 (58.59)	48 (37.50)	5 (3.91)	198 (77.34)	58 (22.66)
Female	165	109 (66.06)	43 (26.06)	13 (7.88)	261 (79.09)	69 (20.91)
Anti-AchR-positive	86	53 (61.63)	30 (34.88)	3 (3.59)	136 (79.07)	36 (20.93)
Anti-AchR-negative	207	131 (63.29)	61 (29.47)	15 (7.25)	323 (78.02)	91 (21.98)
NT-MG group	184	116 (63.04)	52 (28.26)	16 (8.70)	284 (82.07)	84 (17.93)
T-MG group	121	76 (62.81)	37 (30.58)	8 (6.61)	189 (78.10)	53 (21.90)
Male	150	96 (64.00)	46 (30.67)	8 (5.33)	238 (79.33)	62 (20.67)
Female	155	96 (61.94)	43 (27.74)	16 (10.32)	235 (75.81)	75 (24.19)
Anti-AchR-positive	110	77 (70.00)	26 (23.64)	7 (6.36)	180 (81.82)	40 (18.18)
Anti-AchR-negative	195	115 (58.97)	63 (32.31)	17 (8.72)	293 (75.13)	97 (24.87)
Ocular	126	79 (62.70)	37 (29.37)	10 (7.94)	195 (77.38)	57 (22.62)
Generalized	179	113 (63.13)	52 (29.05)	14 (7.82)	278 (77.65)	80 (22.35)

Abbreviations: anti-AchR, acetylcholine receptor antibody; NT-MG, nonthymoma-associated myasthenia gravis; T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor-alpha.

Table 7. Haplotype analyses of TNF- α -1031 T/C, -863C/A and -857C/T between the T-MG and NT-MG groups

Haplotype	T-MG group	NT-MG group	χ^2	P	OR (95% CI)
CCC	41 (0.171)	54 (0.148)	0.625	0.429	1.195 (0.768–1.857)
CTA	8 (0.034)	56 (0.151)	3.345	0.067	1.084 (0.437–2.689)
TCC	118 (0.487)	192 (0.552)	0.744	0.3887	0.867 (0.627–1.199)
TTA	12 (0.050)	45 (0.122)	9.083	0.003	0.375 (0.194–0.725)
TTC	5 (0.020)	37 (0.102)	16.203	< 0.001	0.158 (0.057–0.437)

Abbreviations: CI, confidence interval; NT-MG, nonthymoma-associated myasthenia gravis; OR, odd ratio; T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor-alpha.

Figure 2. Serum TNF- α level detected by ELISA among the NC, T-MG and NT-MG groups. Note: * P < 0.05, compared with the NC group; # P < 0.05, compared with the NT-MG group. TNF- α , tumor necrosis factor-alpha; ELISA, enzyme-linked immunosorbent assay; NC, negative control; T-MG, thymoma-associated myasthenia gravis; NT-MG, nonthymoma-associated myasthenia gravis.

varied frequencies.⁴ Recently, TNF- α promoter polymorphisms have been indicated to be involved in the disease severity of chronic inflammatory disorders including pediatric Crohn's disease.¹⁶ Studies have adapted TNF- α polymorphisms -1031 T/C, -863C/A, -857C/T, -308G/A and -238G/A for various diseases like hepatitis B virus (HBV) infection, hepatocellular carcinoma development and HBV persistence.^{17–19} However, the correlation between TNF- α polymorphisms and MG is still limited, and therefore the present study explored the association of TNF- α -1031 T/C, -857C/T and -863C/A polymorphisms with the

Table 8. Correlations between serum TNF- α level and various genotypes in the T-MG group

Site	Genotype	T-MG group
-1031 T/C	TT	84.33 $\pm$ 10.60
	TC+CC	96.52 $\pm$ 14.09*
-857C/T	CC	84.69 $\pm$ 11.09
	CT+TT	92.36 $\pm$ 14.15*
-863C/A	CC	89.19 $\pm$ 12.39
	CA+AA	90.21 $\pm$ 15.53

Abbreviations: T-MG, thymoma-associated myasthenia gravis; TNF- α , tumor necrosis factor-alpha. * P < 0.05, compared with the TT in -1031 T/C and the CC in -857C/T.

Table 9. Logistic regression analysis of the independent risk factors for T-MG

Factor	P	Exp. (B)	95% CI
Gender	0.678	0.889	0.511–1.548
Age	0.096	0.986	0.970–1.003
Anti-AchR	0.014	2.135	1.164–3.917
Osserman's classification	0.024	0.520	0.295–0.918
-1031 T/C	0.043	1.554	1.014–2.379
-857C/T	< 0.001	3.031	1.932–4.753
TTA	< 0.001	10.462	2.827–38.713
TTC	0.187	2.468	0.646–9.436

Abbreviations: anti-AchR, acetylcholine receptor antibody; CI, confidence interval; T-MG, thymoma-associated myasthenia gravis.

susceptibility of T-MG in Northern Chinese Han patients, and found that TNF- α -1031 T/C and -857C/T polymorphisms and the TTA haplotype could be correlated with the occurrence of T-MG.

Our findings revealed that frequencies of the C allele of -1031 T/C and the T allele of -857C/T are significantly increased in the T-MG group than those in the NT-MG and NC groups, indicating that the C allele of -1031 T/C and the T allele of -857C/T are involved in the susceptibility of T-MG patients. MG is a series of antibody-mediated autoimmune disorders that are T-cell-dependent.²⁰ TNF- α , as a pro-inflammatory cytokine derived from macrophage-monocyte lineage, is involved in driving autoimmunity.²¹ TNF blockade has been reported to regulate B-cell responses, particularly inducing T-cell-dependent humoral immunity.²² The B-cell-activating factor belongs to the TNF family (BAFF), which is crucial to B-cell survival and maturation.^{23,24} Excess BAFF can result in the development of autoimmune disorders and has been detected in various autoimmune conditions.²⁵ Therefore, we speculated that TNF- α might be involved in the susceptibility of T-MG through its correlation with T cells and B cells. Consistently, it was reported that immunoregulatory defects were found in MG patients and were caused by regulatory T-cell impairment, which was supposed to be closely related with TNF- α .²⁶ In addition, elevated TNF- α was demonstrated to be involved in the initiation of myeloproliferative neoplasms and multiple sclerosis.^{27,28} The transcriptional promoter activity of the C allele of -1031 T/C and the T allele of -857C/T in response to concanavalin A stimulation is about twofold greater than that of the dominant allele, suggesting that they might be involved in high levels of TNF- α production in immune responses to multiple stimuli.²⁹ In accordance with our findings, the study in Japanese individuals with TAO, which is generally regarded as an autoimmune disorder, indicated that TNF- α -1073 T/C polymorphisms are consumingly associated with the increased susceptibility of TAO.⁸ Hence, TNF- α -1031 T/C and -857C/T polymorphisms might be associated with T-MG susceptibility. In addition, logistic regression analysis validated that -1031 T/C and -857C/T polymorphisms and TTA haplotype were independent risk factors of T-MG.

In addition, our findings pointed out that the T-MG male patients showed significantly higher frequencies of the CC genotype and the C allele of TNF- α -1031 T/C than those in the NC male patients, indicating that TNF- α -1031 T/C polymorphism was more associated with the male T-MG patients. According to Kamizono *et al.*,⁸ there was no difference in genotype or allele frequencies of any TNF- α polymorphism between male and female patients with Graves' disease (GD), and they did not show any results about the differences between GD and NC patients, possibly because of relative small sample size. TNF- α is a critical mediator of inflammatory and immune functions related with the pathogenesis and progress of inflammatory and autoimmune diseases.^{30,31} TNF- α polymorphisms might affect its production through its transcription, leading to susceptibility of different diseases. Individuals with different TNF- α gene polymorphisms have different levels of plasma TNF- α , which make different immune responses of individuals to the external environment. Considering this, we might have some speculation in this finding, while the accurate mechanisms behind need to be identified with further studies based on gender differences. A previous study has pointed out that the serum TNF- α level was increased in the -1031 T/C genotype carriers, and TNF- α polymorphism correlated with TNF- α level in patients with acute coronary syndrome.³² Giardina *et al.*³³ also demonstrated that TNF- α -857C/T might act as an independent risk factor for psoriatic arthritis, which is consistent with our study. Furthermore, from the study we observed that the patients with ocular MG showed lower frequencies of the TT genotype and the T allele of TNF- α -857C/T than those with generalized MG. Ocular MG, prior to generalized

MG stage, is an initial form of MG that accounts for ~50% of all MG; although there has been a declined rate of ocular MG progressing to generalized MG in MG patients owing to the development of immunomodulation therapies including prednisone therapy, the disease progression is still inevitable.³⁴ According to the analysis above, TNF- α polymorphism -857C allele indicated a high T-MG severity; thus, the ocular MG patients presented higher frequency of TNF- α polymorphism -857 T allele. In addition, we observed that most T-MG patients had B1 or B2 thymoma, and the majority of NT-MG patients had hyperplasia, atrophy of thymus gland or normal thymus gland. Berrih-Aknin *et al.*³⁵ observed in their study that thymic follicular hyperplasia was common in patients with anti-AChR antibodies.

In conclusion, our results found that -857C/T polymorphisms and the TTA haplotype were the independent risk factors of T-MG, as well as anti-AchR and Osserman's classification. Further studies with larger sample size should be further conducted to verify our conclusion.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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