Original Paper

Genetic Association of *MiR-146a* with Multiple Sclerosis Susceptibility in the Chinese Population

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Key Words

MiR-146a • Multiple sclerosis • Polymorphism

Abstract

Background: *miR-146a* polymorphisms have been involved in susceptibility to multiple diseases. The aim of the present study was to analyze the potential association between two functional *miR-146a* polymorphisms (rs2910164 and rs57095329) and multiple sclerosis (MS) in the Han Chinese population. **Methods:** A cohort of 525 patients and 568 healthy controls were genotyped to detect the two polymorphisms by SNaPshot. **Results:** No significant differences were detected in the distribution of the two *miR-146a* polymorphisms between the patients and controls ($P > 0.05$). However, stratification by gender showed a statistically significant difference in the frequency of the genotype rs2910164 between MS patients and control females ($P=0.009$). Further stratification analysis by subgroup revealed that the *miR-146a* rs2910164 C allele conferred a higher risk of developing relapsing–remitting MS (RRMS) ($P=0.018$). In addition, the rs2910164 C allele was significantly associated with increased expression of *miR-146a* in patients with RRMS ($P=0.025$). Moreover, patients with the rs2910164 C allele released more TNF- α and IFN- γ , but not IL-1 β , compared with individuals carrying the homozygous GG genotype ($P < 0.05$). **Conclusions:** Our results provide evidence that rs2910164 may play a role in MS susceptibility in females. The rs2910164 G>C variation may affect the expression of *miR-146a* and the release of proinflammatory cytokines.

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Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating neurodegenerative disease of the central nervous system (CNS) afflicting two million people worldwide [1]. The disease is characterized by infiltration of the CNS with autoreactive immune cells, demyelination, acute astrogliosis, and axonal damage due to immune reactions against the myelin sheath [2]. Approximately 80% of MS patients diagnosed with relapsing–remitting MS (RRMS) develop a secondary, progressive MS course over the following decade. In a smaller group of patients (20%), MS begins with a primary, progressive phase (PPMS) [3]. Although the precise molecular pathogenic mechanism of MS still remains elusive, there is solid evidence that both genetic and environmental factors are implicated in MS susceptibility [4]. The major histocompatibility complex on chromosome 6p21.3 has been well studied, is thought to be strongly associated with MS susceptibility, and has been unambiguously identified in all studied populations [5]. A large body of scientific research indicates that the HLA-DRB1 gene is the main genetic factor for MS. However, the HLA (human leukocyte antigen) genes account for only half of the genetic liability to the disease [6]. Therefore, in recent years, multiple, non-HLA genes have been implicated in susceptibility to MS, but many other genes remain to be discovered.

MicroRNAs (miRNAs) represent a family of small (~22 nucleotides), endogenous, non-coding RNAs that modulate the expression of multiple target mRNAs by inducing either translational inhibition or mRNA degradation. MiRNAs are critically involved in various physiological and pathological processes, including the regulation of innate and adaptive immune responses [7]. One of these conserved miRNAs is miR-146a, which is well known for its powerful regulation of immune response and inflammation. *MiR-146a*, located in the *LOC285628* gene on human chromosome 5, is involved in modulating the negative regulation of Toll like receptor (TLR) signaling, airway remodeling-associated proteins (MMP-13 and collagen II), and pivotal inflammatory cytokines [8]. The dysregulation of miR-146a is associated with multiple autoimmune diseases, such as systemic lupus erythematosus (SLE) [9], rheumatoid arthritis (RA) [10], Sjogren's syndrome (SS) [11], and MS [12]. Recently, miR-146a was found to be upregulated in active MS lesions [13] in humans and in experimental autoimmune encephalomyelitis (EAE) lesions of mice and marmosets [14], which suggests that miR-146a plays a pivotal role in the pathophysiology of MS.

Polymorphisms affecting miRNA expression, maturation, or mRNA recognition may represent an important risk determinant of disease susceptibility. The C allele of the rs2910164 G>C polymorphism of *miR-146a* causes mis-pairing within the *miR-146a* hairpin, decreased expression of its mature form, and abated expression of its target genes, including TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) [15]. This functional polymorphism was most extensively studied for its association with various diseases, including temporal lobe epilepsy [16], asthma [17], SLE [9, 18], and RA [10]. A very recent study also demonstrated that the rs57095329 A>G polymorphism in the promoter region of *miR-146a* was associated with SLE in Chinese individuals, and the mutant allele alleviated miR-146a expression through the reduction of its binding affinity for the transcription factor Ets-1 [18]. To date, there are few studies that have investigated the association between single *miR-146a* polymorphisms and MS susceptibility, and the only case-controlled study for *miR-146a* rs2910164 performed in Caucasians failed to find any differences in the frequencies of rs2910164 between patients and controls [19]. Given the critical regulatory role of miR-146a in immune response and its elevation in MS, it is likely that polymorphisms in the *miR-146a* gene could potentially confer disease risk. In the present study, we performed a case-controlled study to ascertain whether the two polymorphisms within the *miR-146a* gene are associated with MS susceptibility in the Chinese population and whether these polymorphisms are associated with miR-146a expression and proinflammatory cytokine production.

Materials and Methods

Participant recruitment

Our study consecutively recruited 525 patients (276 female, 103 male) with clinically definite MS according to the criteria of McDonald and colleagues [20] from the Department of Neurology at the first affiliated hospital and the fourth affiliated hospital of Harbin Medical University in northern China. Disease severity was evaluated using the Kurtzke Expanded Disability Status Scale (EDSS).

Disease duration was defined as the time interval between disease onset and the most recent hospitalization. The progression index (PI), defined as the EDSS/ disease duration (years), was calculated if disease duration was ≥ 5 years. According to Lublin's criteria, 379 MS patients were categorized as having RRMS, 109 of the patients were categorized as having SPMS and 37 MS patients were categorized as having PPMS. The patients' clinical characteristics are summarized in Table 1. A total of 568 unrelated healthy blood donors (376 female, 192 male; mean age 32.5 ± 10.7 years), matched for ethnicity, age and gender, and whose families had no history of MS or any other inflammatory-demyelinating disease, were recruited from the same geographical area in the same period as control subjects. Written informed consent was obtained from all of the enrolled participants. Ethical approval was obtained for the study from the relevant ethics committees.

DNA extraction and genotyping

Genomic DNA was extracted from whole blood samples from all of the patients and controls using the TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. A total of 1093 individuals were genotyped for the two single-nucleotide polymorphisms (SNPs) (rs2910164 G/C and rs57095329 A/G) using the SNaPshot technique (Applied Biosystems, Foster City, CA, USA). The PCR primers used in the SNaPshot were as follows: rs2910164F: GAACTGAATTCCATGGGTTG, rs2910164R: CACGATGACAGAGATATCCC; rs57095329F: TCATTGGGCAGCCGATAAAG, rs57095329R: AGGAAGTTCTGGTCAGGCG. Genotyping was conducted by polymerase chain reaction (PCR) according to the manufacturer's protocol as it is described previously [21]. The final data were analyzed using GeneMapper 4.1 (Applied Biosystems, Foster City, CA, USA).

MiRNA extraction and real-time PCR

To ascertain whether the genotypes of *miR-146a* polymorphisms (rs2910164 and rs57095329) correlated with differences in gene expression, the levels of *miR-146a* expression were determined by real-time PCR in peripheral blood mononuclear cells (PBMC) from 50 RRMS patients and 50 controls. PBMCs were isolated using density gradient centrifugation method with LymphoprepTM (Axis-Shield PoCAS, Oslo, Norway) as we described previously [21]. MiRNA extracted from PBMCs was performed using the miRcute miRNA Isolation Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The quantity of mature *miR146a* was determined by qRT-PCR using the SYBR green method as we described previously [22]. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. Values were normalized to *snRNA U6*.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples collected for the measurement of plasma cytokines concentrations were drawn in EDTA-containing tubes. The plasma aliquots were obtained by centrifuging the blood samples at low speed. Levels of TNF- α , IFN- γ and IL-1 β were measured in duplicate using Quantikine sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were conducted using SPSS, version 19.0 (IBM, NY, USA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Allele frequencies and genotype distribution of MS patients and controls were compared using the Fisher's exact test or Chi-squared test. Deviation of the genotype or allele frequency was assessed using Hardy-Weinberg equilibrium (HWE). Analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) were used to compare the relationships between the genotypes of *miR-146a* polymorphisms and the onset age or PI of MS patients. The non-parametric Mann Whitney U test was used to compare the *miR-146a* expression and the production of TNF- α , IFN- γ , and IL-1 β level among three genotypes of rs2910164 and rs57095329, respectively. The criterion for significance was set at $P < 0.05$ for all the tests.

Table 1. Characteristics of MS cases and controls. Continuous data are presented as mean $\pm$ SD. MS: multiple sclerosis, EDSS: Expanded Disability Status Scale, PI: Progression Index, PPMS: primary progressive MS, RRMS: relapsing-remitting MS, SD: standard deviation, SPMS: secondary progressive MS

Variables	MS patients (n=525)			controls (n=568)
	RRMS (n=379)	SPMS (n=109)	PPMS (n=37)	
Mean age at onset (years)	32.6 $\pm$ 10.3	32.1 $\pm$ 9.6	33.1 $\pm$ 11.8	32.5 $\pm$ 10.7
Female/male	276/103	78/31	27/10	376/192
Mean duration	11.5 $\pm$ 7.8	10.8 $\pm$ 10.5	11.2 $\pm$ 9.8	-
Mean EDSS	3.9 $\pm$ 3.1	4.0 $\pm$ 2.6	4.2 $\pm$ 3.2	-
Mean PI	0.42 $\pm$ 0.32	0.45 $\pm$ 0.28	0.48 $\pm$ 0.32	-

Table 2. Frequencies of *miR-146a* genotypes and alleles in MS patients and controls

Genotypes	MS patients n=525 (%)	Controls n=568 (%)	OR (95%CI)	P value
rs2910164				
GG	91(17.3)	119(21.0)		0.31
GC	252(48.0)	257(45.2)		
CC	182(34.7)	192(33.8)		
Dominant model GG/GC vs CC	343(65.3)	376(66.2)	0.96(0.75-1.24)	0.76
Recessive model GG vs GC/CC	434(82.7)	449(79.0)	0.79(0.58-1.07)	0.13
G allele	434(41.3)	495(43.6)	1.000(reference)	
C allele	616(58.7)	641(56.4)	0.91(0.77-1.08)	0.29
rs57095329				
AA	332(63.2)	337(59.3)		0.22
AG	171(32.6)	196(34.5)		
GG	22(4.2)	35(6.2)		
Dominant model AA/AG vs GG	503(96.6)	533(93.8)	1.50(0.87-2.59)	0.14
Recessive model AA vs AG/GG	193(34.9)	231(40.7)	1.18(0.92-1.51)	0.19
A allele	835(80.9)	870(76.6)	1.000(reference)	
G allele	215(19.1)	266(23.4)	1.19(0.97-1.46)	0.10

Results

Characteristics of the study population

The characteristics of the MS patients and control groups are presented in Table 1. The cases and controls were matched according to age and sex.

Genotype and allele frequencies of miR-146a polymorphisms in MS patients and controls

The genotype distribution and allele frequencies of the *miR-146a* polymorphisms in all patients with MS and the control group are presented in Table 2. No deviation from Hardy-Weinberg equilibrium was observed in the genotype distributions of the MS patients and controls (data not shown). The overall genotype and allele distributions of the *miR-146a* rs2910164 and rs57095329 polymorphisms did not differ significantly between MS patients and controls ($P>0.05$) (Table 2).

Table 3. Frequencies of *miR-146a* genotypes and alleles stratified by gender in MS patients and controls

Genotypes	Female		OR (95%CI)	P value	Male		OR (95%CI)	P value
	MS patients n=381(%)	Controls n=376(%)			MS patients n=144(%)	Controls n=192(%)		
rs2910164								
GG	50(13.1)	81(21.5)		0.009	23(16.0)	40(20.8)		0.44
GC	193(50.7)	172(45.7)			64(44.4)	86(44.8)		
CC	138(36.2)	123(32.7)			57(39.6)	66(34.4)		
Dominant model GG/GC vs CC	243(63.9)	253(67.3)	0.86(0.64-1.17)	0.33	87(60.4)	126(65.6)	0.80(0.51-1.25)	0.33
Recessive model GG vs GC/CC	331(86.8)	295(78.5)	0.55(0.37-0.81)	0.002	121(84.0)	152(79.2)	0.72(0.41-1.27)	0.26
G allele	293(38.5)	334(44.4)	1.000(reference)		110(38.2)	166(43.2)	1.000(reference)	
C allele	469(61.5)	418(55.6)	0.78(0.64-0.96)	0.019	178(61.8)	218(56.8)	0.81(0.59-1.11)	0.19
rs57095329								
AA	246(64.6)	223(59.3)		0.32	94(65.3)	110(57.3)		0.33
AG	117(30.7)	131(34.8)			45(31.3)	74(38.5)		
GG	18(4.7)	22(5.9)			5(3.4)	8(4.2)		
Dominant model AA/AG vs GG	363(95.3)	354(94.1)	1.25(0.66-2.38)	0.49	139(96.5)	184(95.8)	1.21(0.39-3.78)	0.74
Recessive model AA vs AG/GG	135(35.4)	153(40.7)	1.25(0.93-1.68)	0.14	50(34.7)	82(42.7)	1.40(0.90-2.19)	0.14
A allele	609(79.9)	577(76.7)	1.000(reference)		233(80.9)	294(76.6)	1.000(reference)	
G allele	153(20.1)	175(23.3)	1.21(0.95-1.54)	0.13	55(19.1)	90(23.4)	1.30(0.89-1.89)	0.18

Table 4. Clinical characteristics according to *miR-146a* genotype status. Continuous data are presented as mean $\pm$ SD. EDSS: Expanded Disability Status Scale, PI: Progression Index

Clinical features	<i>miR-146a</i> rs2910164		P value	<i>miR-146a</i> rs57095329		P value
	GG	GC/CC		AA	AG/GG	
No. of subjects	91	434		342	183	
Most recent EDSS	3.8 $\pm$ 2.5	4.1 $\pm$ 2.6	0.56	3.9 $\pm$ 2.2	4.0 $\pm$ 1.5	0.78
PI	0.41 $\pm$ 0.22	0.50 $\pm$ 0.48	0.038	0.46 $\pm$ 0.36	0.49 $\pm$ 0.32	0.55
Age of onset, years	31.5 $\pm$ 10.8	33.6 $\pm$ 9.8	0.81	32.2 $\pm$ 9.5	33.2 $\pm$ 11.2	0.25

Table 5. Association between the *miR-146a* genotypes and alleles and MS subtypes

	rs2910164			P value	rs57095329			P value
	Genotype GG GC CC	Allele G C	P value		Genotype AA AG GG	Allele A G	P value	
Controls	119 257 192 (21.0) (45.2) (33.8)	495 641 (43.6) (56.4)			337 196 35 (59.3) (34.5) (6.2)	870 266 (76.6) (23.4)		
Cases								
RRMS	49 191 139 (12.9) (50.4) (36.7)	289 469 (38.1) (61.9)	0.007	0.018	245 119 15 (64.6) (31.4) (4.0)	609 149 (80.3) (19.7)	0.15	0.053
SPMS	33 44 32 (30.3) (40.4) (29.4)	110 108 (50.5) (49.5)	0.10	0.06	73 34 2 (67.0) (31.2) (1.8)	180 38 (82.6) (17.4)	0.12	0.052
PPMS	9 17 11 (24.3) (45.9) (29.7)	35 39 (47.3) (52.7)	0.83	0.53	24 12 1 (64.9) (32.4) (2.7)	60 14 (81.1) (18.9)	0.63	0.37

Stratification of genotype and allele frequencies of *miR-146a* polymorphisms in MS patients and controls by gender

When the MS patients and controls were stratified by gender, a significant difference was found in the frequency of the rs2910164 GC and CC genotype of *miR-146a* between female controls and female MS patients ($P = 0.009$). Additionally, a significant increase in the risk of MS was observed with a recessive model (OR=0.55, 95% CI: 0.37-0.81, $P = 0.002$) (Table 3). The *miR-146a* rs57095329 genotypes and alleles were not significantly associated with the risk of developing MS for either male or female individuals.

Effect of *miR-146a* gene polymorphisms on clinical characteristics

The general characteristics of the subjects according to their *miR-146a* rs2910164 and rs57095329 genotypes are shown in Table 4. The age of onset and EDSS was not significantly associated with the presence of the variant genotypes (GC/CC or AG/GG). However, our data showed that rs2910164 GC/CC carriers had a higher PI than did GG allele homozygotes ($P = 0.038$) (Table 4).

Fig. 1. (A) Mean relative miR-146a values $\pm$ SD in PBMCs from RRMS patients (RRMS, n=50) and healthy control subjects (controls, n=50). The blank box and the black box represent the relative expression of miR-146a in the controls and the RRMS patients, respectively. *P=0.027 when comparing relative miR-146a levels between RRMS patients and controls. (B) Mean relative miR-146a values $\pm$ SD in PBMCs from RRMS patients (RRMS, n=50) and healthy control subjects (controls, n=50) stratified by gender. The blank box and the black box represent the relative expression of miR-146a in male and female individuals, respectively. *P=0.042 when comparing relative miR-146a levels between male and female patients with RRMS.

Association of miR-146a gene polymorphisms with MS subtypes

Because MS manifests as different disease subtypes, it is important to test whether *miR-146a* gene polymorphisms act as modifiers of the disease course. A statistically significant difference in the *miR-146a* rs2910164 GC and CC genotype and C allele carrier frequency between patients and controls was identified in the RRMS subgroup (P=0.007 for genotype and P=0.018 for allele) (Table 5). However, the number of primary, progressive MS patients was too small to draw firm conclusions. We did not find any significant association of the *miR-146a* rs57095329 genotypes and alleles with the risk of developing MS in any MS subtypes (RRMS, SPMS or PPMS).

Effect of miR-146a gene polymorphisms on miR-146a expression

A comparative determination was performed on miR-146a expression in the PBMCs of 50 RRMS patients and 50 healthy controls. Consistent with a previous report [19], the mean value of miR-146a expression was significantly higher in the RRMS patients than in the controls (P=0.027) (Fig. 1A). We also determined the miR-146a expression levels in the patients and controls stratified by gender. An increased expression of miR-146a was detected in female patients with RRMS (P=0.042; Fig. 1B). However, in the normal control group, the expression of miR-146a was indistinctive in female or male individuals (Fig. 1B). Previous studies have suggested that common GC polymorphisms might alter the free binding energy of the pre-miR-146a, which would affect the miRNA-processing efficiency [15]. To identify the effect of *miR-146a* polymorphisms on miR-146a expression, real-time PCR was performed to determine the expression levels of miR-146a in PBMCs of both RRMS patients and controls. The results are presented in Fig. 2. A significant increase in miR-146a expression was observed in RRMS patients who carried the mutated rs2910164 GC (P=0.028) or CC (P=0.035) genotypes. Comparatively, a significant decrease in miR-146a expression was detected in RRMS patients who carried the mutated rs57095329 AG (P=0.038) or GG (P=0.022) genotypes. However, no statistically significant difference in miR-146a expression was found between the population carrying the mutated allele (GC or CC for rs2910164 and AG or GG for rs57095329) and the population carrying the major GG or AA in the control subjects (P>0.05) (Fig. 2).

Fig. 2. Mean miR-146a values $\pm$ SD in PBMCs isolated from the RRMS patients (n=50) and controls (n=50) stratified according to three different genotypes of rs2910164 and rs57095329, respectively. Real-time PCR analysis of mature miR-146a expression in PBMCs derived from the RRMS patients and healthy individuals with rs2910164 GG, GC and CC genotype and rs57095329 AA, AG and GG genotypes. Asterisk indicates $P < 0.05$.

Fig. 3. Proinflammatory cytokines production with three different genotypes of rs2910164 in PBMCs. TNF- α (A), IFN- γ (B) and IL-1 β (C) in plasma of PBMCs derived from the RRMS patients and healthy individuals with rs2910164 GG, GC and CC genotypes were measured by ELISA. Data are shown as mean $\pm$ SD. Asterisk indicates $P < 0.05$.

The influence of rs2910164 polymorphism on cytokine production

Another experiment was performed to determine whether the *miR-146a* rs2910164 polymorphism had an effect on proinflammatory cytokine production in controls and RRMS patients. The results are shown in Fig. 3. Generally, the plasma expression levels of TNF- α , IFN- γ , and IL-1 β were significantly in RRMS patients than in the controls ($P < 0.05$) (Fig. 3). Furthermore, the RRMS patients carrying the mutated GC or CC genotype presented with higher plasma TNF- α ($P = 0.036$ for GC and $P = 0.024$ for CC) and IFN- γ ($P = 0.015$ for GC and $P = 0.008$ for CC) levels compared to the homologous GG group (Fig. 3A and 3B). The expression of IL-1 β was not affected when stratified by different genotypes, in either RRMS patients or controls ($P > 0.05$) (Fig. 3C).

Discussion

In this hospital-based, case-control study, we demonstrate for the first time that the rs2910164 polymorphism of *miR-146a* is associated with MS in female patients and that the rs57095329 polymorphism in the promoter region of *miR-146a* is not associated with MS. Further stratification revealed that the rs2910164 G>C polymorphism of *miR-146a* is associated with RRMS. Additionally, the *miR-146a* rs2910164 G>C variation modulates the expression of miR-146a and the release of TNF- α and IFN- γ .

MiRNAs have been recognized as important regulators in common human disorders. miR-146a is a microRNA that regulates innate immune function, inflammatory responses

and antiviral pathways. MiR-146a may act as a negative feedback regulator of the immune response by targeting two genes, TRAF6 and IRAK1, which are crucial for proinflammatory signaling [23]. In addition, miR-146a is important for the function of regulatory T cells, and a deficiency of this miRNA results in a defect in their suppressor function and dysregulated IFN- γ responses [24]. Recently, Lescher et al. found that miR-146a expression was increased in EAE lesions of mice and marmosets [14]. miR-146a expression was also significantly elevated in the PBMCs of RRMS patients compared to healthy controls [19, 25]. This evidence suggests that miR-146a plays an important role in MS pathogenesis. Despite these advances, *miR-146a* alleles that contribute to MS pathology remain undiscovered.

Polymorphisms affecting miRNA expression, maturation, or mRNA recognition may also become important determinants for an increased risk of developing MS [9]. Several studies have examined the association between the *miR-146a* rs2910164 polymorphism and autoimmune diseases, including RA [8], SLE [8, 9] and MS [19]. Although previous studies have suggested that many autoimmune diseases share common predisposing factors, the results are inconsistent [26]. In this hospital-based, case-control study, the allele and genotype frequencies of the *miR-146a* polymorphisms did not differ significantly between the MS patients and healthy controls; this result is consistent with Fenoglio's previous report [19]. Interestingly, in our population, the rs2910164 allele frequency was 56.4% for the C allele, which is significantly higher than the rs2910164 allele frequency (25.0%) in Caucasians [19]. This discrepancy might result from profound ethnic differences.

However, we did find an association between the rs2910164 polymorphism and MS risk in the female subgroup of MS patients and controls. MS is universally more prevalent in females. There is evidence that gender affects the prevalence, clinical course, pathology, and prognosis of MS. It is believed that sex hormones, especially estrogen, play an important role in MS. Estrogen promotes the secretion of proinflammatory cytokines, such as IFN- γ and IL-17 [27], either through the modification of key transcription factors in inflammation or through the regulation of miRNA expression. When splenic lymphocytes are treated with estrogen, miR-146a expression decreases [28]. This decrease may cause T-cells to become hyperactive in a chronic inflammatory autoimmune response. Other than hormones, several other factors are likely to be especially relevant in MS pathogenesis between male and female patients, including intrinsic biological differences in male and female immune systems, nervous systems, genetic and epigenetic factors, environmental exposure, and maternal microchimerism [29]. In this study, the observation that females have a higher MS prevalence than males may result from an interaction between the genotype and sex hormones during development. Gender-dependent associations have been shown in other case-control studies involving immune entities [8]. As the *miR-146a* gene is located on 5q33.3, one of the richest loci for genes associated with autoimmune diseases, the linkage disequilibrium between rs2910164 and SNPs near *miR-146a* (such as rs2431697 and rs2277920) or in other candidate genes (*ADRB2*, *IL-12*, *IL-4*, *IL-13*) should also be taken into consideration.

MiR-146a negatively modulates innate immunity through the regulation of TLR signaling and cytokine responses by targeting TRAF6 and IRAK1 [23]. In RRMS, inflammatory events play a pivotal role and occur in response to an aberrant activation of the immune system towards myelin proteins, which are supposed to act as autoantigens [19]. However, in progressive forms of MS, neurodegeneration is prominent. This neurodegeneration is demonstrated by the development of motor disabilities, cognitive deficits and brain atrophy. It is conceivable that the dysregulation of miRNAs specific to the activation and differentiation of the immune system is predominant in the acute phase of the disease. In this study, we found that the *miR-146a* rs2910164 GC and CC genotype and C allele are associated with RRMS. This association may occur because individuals with the rs2910164 GC and CC genotype express higher levels of miR-146a. It is likely that the rs2910164 G>C variation is linked with stronger inflammatory responses, and this hypothesis is supported by our findings that the rs2910164 GC and CC genotype is associated with higher TNF- α and IFN- γ levels in RRMS patients.

MiRNA expression analysis revealed a dysregulation of certain miRNAs in patients compared to controls. Increased miR-146a has been found in other autoimmune diseases, such as RA, SLE, and SS [12, 19]. In the present study, we determined that miR-146a expression was significantly elevated in the PBMCs of MS patients compared to controls. This result is correlated with Fenoglio's report [19]. We also revealed that RRMS patients carrying the mutated rs2910164 GC and CC genotype have higher expression of miR-146a than those with the GG genotype. However, whether the rs2910164 C allele was associated with increased or decreased miR-146a expression level is rather inconsistent. Some previous studies indicated that the rs2910164 G>C variation causes mis-pairing within the *miR-146a* hairpin, thus leading to decreased expression of its mature form [15, 30, 31]. Nevertheless, other studies observed that the rs2910164 CC genotype was linked with increased miR-146a in breast/ovarian cancer patients [30] or coronary artery disease patients [32, 33]. The discrepancy between these studies is intriguing, although the exact molecular mechanism of how this G>C variation might affect the mature miRNA expression remains to be determined. Considering the effects of increased miR-146a expression on MS development, it is conceivable that individuals with C allele of rs2910164 in *miR-146a* may have increased miR-146a expression, which may enhance the immune and inflammatory responses, thereby contributing to increased MS risk.

The neuroinflammatory process plays a significant role in MS development, and inflammatory reactions with myeloid cells may be primordial and a possible target in MS. TNF- α is associated with clinical activity in RRMS and the development of progressive disease. High levels of TNF- α have been found in the blood and cerebrospinal fluid (CSF) of MS patients [34]. Microarray analyses of MS lesions have also demonstrated increased transcripts of genes encoding for inflammatory cytokines, particularly IL-6, IL-17, and IFN- γ [35]. In the present study, we evaluated the effect of the rs2910164 polymorphism on plasma TNF- α , IFN- γ and IL-1 β expression. Our data revealed that individuals carrying the GC and CC genotype of rs2910164 had a higher expression of TNF- α and IFN- γ in RRMS patients, and the rs2910164 G>C variation had no effect on IL-1 β production. Because miR-146a negatively regulates inflammatory responses, MS patients should show decreased production of inflammatory or proinflammatory cytokines, such as TNF- α , IFN- γ , or IL-1 β , if miR-146a expression is increased. However, we observed the inverse phenomenon in the present study. These findings imply that miR-146a might fail to accurately regulate its targets in MS due to unclear mechanisms and might lead to inefficient clearance of self-antigens and inflammation protracted *in vivo*, which eventually results in chronic inflammation and the activation of adaptive immune responses. Therefore, based on current knowledge, more investigations are still needed to determine the connection between miR-146a and MS.

There are several important limitations to this case-control study. First, the limited power of the present sample for the case-control study may have suppressed a true relationship due to a type II error. However, notably, the prevalence of MS in the Chinese population is lower than in the Caucasian population [1]. The mechanism that underlies this interesting ethnic difference remains unclear. Moreover, the connection between miR-146a polymorphisms and MS subtype should be interpreted with caution, because the number of SPMS and PPMS samples analyzed was quite small. Thus, these preliminary findings are not definitive and must be replicated in a larger population. Second, selection bias in the patient or control populations cannot be entirely excluded. The current results should be interpreted with caution due to the limited number of male patients. In addition, we only analyzed the effect of the *miR-146a* polymorphisms on miR-146a expression and proinflammatory cytokine production in RRMS patients because a positive association was found between the rs2910164 polymorphism and MS in RRMS patients and controls. Third, other risk factors in the study group, such as Epstein-Barr Virus infection, smoking and vitamin D deficiency, may have complicated the association between the *miR-146a* polymorphisms and MS. Finally, other functional polymorphisms may influence the expression of miR-146a or contribute to MS risk, and their combined effects must be studied to better predict the occurrence, severity, and outcome of MS. The results obtained in this study require confirmation in an

independent study with larger populations and in different ethnic backgrounds before the conclusions can be considered definitive and useful for estimating an individual's risk for developing MS.

In conclusion, our study shows a novel, significant association between the *miR-146a* rs2910164 polymorphism and an increased risk of MS in female patients. Furthermore, our findings suggest that this gene polymorphism may affect the genetic predisposition to this disease through modulating the expression of mature miR-146a and influencing the release of proinflammatory cytokines in MS patients and controls. This new knowledge about miR-146a may be clinically important and confirms a role for miR-146a in the pathophysiology of MS, with potentially important therapeutic implications.

Disclosure Statement

The authors have no actual or potential conflicts of interest related to this manuscript. Appropriate approval was obtained, and appropriate procedures were followed concerning human subjects.

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