Variant of *EOMES* Associated with Increasing Risk in Chinese Patients with Relapsing-remitting Multiple Sclerosis

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Abstract

Background: Multiple sclerosis (MS) is a common central nervous system autoimmune disorder. Increasing number of genome-wide association study (GWAS) analyses hint that MS is strongly associated with genetics. Unfortunately, almost all the GWAS analyses were Caucasian population based. Numbers of risk loci might not be replicated in Chinese MS patients. Hence, we performed a MassArray Assay to genotype the previously reported variants located in the transcription regulation genes in order to elucidate their role in the Chinese MS patients.

Methods: One hundred and forty-two relapsing-remitting MS (RRMS) patients and 301 healthy controls were consecutively collected from September 2, 2008, to June 7, 2013, as stage 1 subjects. Eight reported transcription regulation-related single-nucleotide polymorphisms (SNPs) were genotyped using the Sequenom MassArray system. In stage 2, another 44 RRMS patients and 200 healthy controls were consecutively collected and Sanger sequenced from April 7, 2015, to June 29, 2017, for the validation of positive results in stage 1. Differences in allele and genotype frequencies between patients and healthy controls, odds ratios, and 95% confidence intervals were calculated with the Chi-square test or Fisher's exact test. Hardy-Weinberg equilibrium was tested also using the Chi-square test.

Results: In stage 1 analysis, we confirmed only one previously reported risk variant, rs11129295 in *EOMES* gene. We found that the frequency of T/T genotype was much higher in MS group ($\chi^2 = 10.251$, $P = 0.005$) and the T allele of rs11129295 increased the risk of MS ($\chi^2 = 10.022$, $P = 0.002$). In stage 2 and combined analyses, the T allele of rs11129295 still increased the risk of MS ($\chi^2 = 4.586$, $P = 0.030$ and $\chi^2 = 16.378$, $P = 5.19 \times 10^{-5}$, respectively).

Conclusions: This study enhances the knowledge that the variant of *EOMES* is associated with increasing risk in Chinese RRMS patients and provides a potential therapeutic target in RRMS.

Key words: Genetic Association Studies; Multiple Sclerosis; Risk Factors; Single-nucleotide Polymorphism

Introduction

Multiple sclerosis (MS; MIM 126200) is a common, partially heritable central nervous system (CNS) autoimmune disorder being considered as one of the most common causes of neurological disability in young adults.[1,2] Relapsing-remitting MS (RRMS) is the most common form of MS which has a biphasic disease course characterized by alternating episodes of acute neurological deficits, followed by a complete or partial recovery. Although the exact pathogenesis of MS is still not well classified, it is no doubt that genetic factors are primarily responsible for the substantially increased frequency of the disease, especially in the relatives of affected individuals.[3-5] For years, the major histocompatibility complex (MHC) was the only known MS susceptibility region.[6] However, hundreds of variants outside MHC region had now been identified by several genome-wide association studies (GWASs).[7,8]

The major pathogenic process of MS mainly starts with the activation of autoreactive lymphocytes and their migration across the blood-brain barrier. Transcription factors (TFs)

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are a group of key regulators involving in controlling lineage differentiation and cell survival. Certainly, TFs are indispensable for the T helper (Th) cell fate determination and cytokine production. A number of previous studies have demonstrated that the dysregulation of TFs is deeply involved in the pathogenesis of MS.[9-11] In addition, a series of GWAS analyses had discovered several risk loci within transcription regulation genes. In 2011, a Caucasian population-based multicenter GWAS analysis reported at least eight transcription regulator-related risk loci among 29 novel MS susceptibility loci.[7] Later on, another GWAS analysis replicated these results in a larger cohort.[8] In addition, it has been indicated that the mRNA expression of one TF, *EOMES*, was significantly and consistently lower in MS patients comparing to healthy controls (HCs).[12]

However, to date, few analyses were conducted to identify the variants located in transcription regulation genes in Asian MS populations. Here, we performed a MassArray Assay to genotype the previously reported variants located in the transcription regulation genes in order to elucidate their role in the Chinese MS patients.

METHODS

Ethical approval

The written informed consent was signed by each participant before inclusion in the study. This study was approved by the Ethics Committee of Huashan Hospital of Fudan University, the Second Affiliated Hospital of Zhejiang University School of Medicine, and the First Affiliated Hospital of Fujian Medical University.

Subjects

In the first stage, a total of 142 MS patients (59 males, 83 females; mean age 39.8 ± 12.3 years; range: 13.0–66.0 years) were consecutively collected from September 2, 2008, to June 7, 2013. All patients underwent detailed neurological examinations, laboratory tests, and magnetic resonance imaging scans for the brain and/or spinal cord. Patients were then diagnosed according to the revised McDonald criteria.[13,14] In addition, 301 healthy individuals (175 males, 126 females; mean age 37.0 ± 15.6 years; range 16.0–85.0 years) with no history of autoimmune diseases were recruited as HCs matched for case ethnicity and region. To validate the positive result from the first stage, another 44 RRMS patients and 200 HCs were consecutively collected from April 7, 2015, to June 29, 2017.

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cell using a QIAamp DNA Blood Minikit (QIAGEN, Hilden, Germany). Eight previously reported TF-related variants were genotyped using the Sequenom MassArray system. MassArray Assay Design 3.1 software (Sequenom, San Diego, USA) was used to design the polymerase chain reaction primers used in the genotyping [Supplementary Table 1]. Alleles were detected using a matrix-assisted laser desorption/ionization time of flight mass spectrometry platform (MassArray™, Sequenom Inc.,

San Diego, CA, USA) according to a previously described method.[15] In validation of the first stage, rs11129295 was further Sanger sequenced in subjects recruited for the second stage using a pair of in-house designed primer (Forward: 5'-TCTTGTTTTCTGGAGAGGAGC-3'; Reverse: 5'-ACCCACCTTCAGGAATTTCAAT-3').

Statistical analyses

Quantitative measures were summarized with descriptive statistics, such as mean ± standard deviation (SD) and 95% confidence interval (*CI*) of mean. Difference in ages was measured using the Student's *t*-test. Differences in genders, allele and genotype frequencies between MS and HC, odds ratios (*OR*s), and 95% *CI*s were calculated using the Chi-square test or Fisher's exact test. Hardy-Weinberg equilibrium (HWE) was tested using the Chi-square test. Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.02 (GraphPad Inc, San Diego, CA, USA). The criterion for a signifcant difference was a value of $P < 0.05$.

RESULTS

Demographic data

The demographic data are summarized in Table 1. In stage 1, the average ages of the MS patients and HCs were 39.8 ± 12.3 and 37.0 ± 15.6 years, respectively ($t = 1.840$, $P = 0.070$). In stage 2, the average ages of the MS patients and HCs were 39.0 ± 13.8 and 33.3 ± 11.3 years, respectively ($t = 2.740$, $P = 0.007$). In total, the average ages of the MS patients and HCs were 39.6 ± 12.5 and 35.5 ± 14.2 years, respectively ($t = 3.310$, $P = 0.001$).

EOMES variant rs11129295 associated with increasing risk in Chinese multiple sclerosis patients

The average genotyping success rate across the single-nucleotide polymorphisms (SNPs) was 97.5% using the Sequenom MassArray system. As shown in Supplementary Table 2, most of the SNPs in each group were under the HWE except *STAT3* variant rs744166 in MS patients ($P = 0.020$). Eventually, as summarized in Table 2, we found that the frequency of T/T genotype in *EOMES* variant rs11129295 was much higher in MS group with a significant difference ($\chi^2 = 10.251$, $P = 0.005$). In addition, the T allele of rs11129295 increased the risk of MS ($OR = 1.764$, $P = 0.002$). Besides, when analyzed in a dominant model, the genotype T/T and C/T together can increase the risk of MS ($OR = 4.076$, $P = 0.019$). Hence, it was a recessive model ($OR = 1.776$, $P = 0.007$). Furthermore, we replicated these findings in another group of population using Sanger sequencing. In this stage, we still found that the frequency of T/T genotype in rs11129295 was much higher in MS group with a significant difference ($P = 0.020$). Besides, the T allele increased the risk of MS ($OR = 1.830$, $P = 0.030$). However, we did not find significance in recessive model in stage 2 [Table 2].

In combined analyses of the two stages, we found that the frequency of T/T genotype in rs11129295 was much higher

Table 1: Demographic and clinical characteristics of RRMS patients and healthy controls

Characteristics	MS	HC	Statistics	P
Stage 1, *N*	142	301		
Male/female, *n*	59/83	175/126	10.656*	0.001
Age (year), mean ± SD	39.8 ± 12.3	37.0 ± 15.6	1.840†	0.070
Stage 2, *N*	44	200		
Male/female, *n*	17/27	98/102	1.555*	0.210
Age (year), mean ± SD	39.0 ± 13.8	33.3 ± 11.3	2.740†	0.007
Stage 1 + Stage 2, *N*	186	501		
Male/female, *n*	76/110	273/228	10.083*	0.001
Age (year), mean ± SD	39.6 ± 12.5	35.5 ± 14.2	3.310†	0.001

*χ^2; †*t*. MS: Multiple sclerosis; RRMS: Relapsing-remitting MS; HC: Healthy controls; SD: Standard deviation.

Table 2: Allele and genotype distributions of rs11129295 between MS and healthy controls

SNP	Region	Candidate Gene	Genotype/Allele	MS, *n* (%)	HC, *n* (%)	χ^2	OR (95% CI)	P
Stage 1								
rs11129295	Intergenic	*EOMES*	TT	91 (65.9)	157 (52.2)	10.251		0.005
			CT	44 (31.9)	119 (39.5)			
			CC	3 (2.2)	25 (8.3)			
			T	226 (81.9)	433 (71.9)	10.022	1.764 (1.238–2.514)	0.002
			C*	50 (18.1)	169 (28.1)			
			CT + TT†	135 (97.8)	276 (91.7)	5.958	4.076 (1.209–13.739)	0.019
			CC	3 (2.2)	25 (8.3)			
			TT‡	91 (65.9)	157 (52.2)	7.313	1.776 (1.169–2.769)	0.007
			CT + CC	47 (34.1)	144 (47.8)			
Stage 2								
rs11129295	Intergenic	*EOMES*	TT	26 (59.1)	97 (48.5)	6.336		0.020
			CT	18 (40.9)	78 (39)			
			CC	0 (0)	25 (12.5)			
			T	70 (79.5)	272 (68.0)	4.586	1.830 (1.046–3.200)	0.030
			C*	18 (20.5)	128 (32.0)			
			CT + TT†	44 (100)	175 (87.5)	6.128	1.143 (1.085–1.204)	0.011
			CC	0 (0)	25 (12.5)			
			TT‡	26 (59.1)	97 (48.5)	1.618	1.534 (0.791–2.973)	0.203
			CT + CC	18 (40.9)	103 (51.5)			
Stage 1 + 2								
rs11129295	Intergenic	*EOMES*	TT	117 (64.3)	254 (50.7)	17.452		4.17×10⁻⁵
			CT	62 (34.1)	197 (39.3)			
			CC	3 (1.6)	50 (10.0)			
			T	296 (81.3)	705 (70.4)	16.378	1.834 (1.363–2.466)	5.19×10⁻⁵
			C*	68 (18.7)	297 (29.6)			
			CT + TT†	179 (98.4)	451 (90.0)	12.947	6.615 (2.037–21.481)	8.12×10⁻⁵
			CC	3 (1.6)	50 (10.0)			
			TT‡	117 (64.3)	254 (50.7)	9.932	1.750 (1.233–2.484)	0.002
			CT + CC	65 (35.7)	247 (49.3)			

*The C allele is the ancestral allele according to dbSNP build 141; †Analysis in a dominant model; ‡Analysis in a recessive model. SNP: Single-nucleotide polymorphisms; MS: Multiple sclerosis; HC: Healthy controls. CI: Confidence interval; OR: Odds ratio.

in MS group with a significant difference ($P = 4.17 \times 10^{-5}$), as well as the T allele increased the risk of MS ($OR = 1.834$, $P = 5.19 \times 10^{-5}$) and the higher frequency of T/T plus C/T in dominant model ($OR = 6.615$, $P = 8.12 \times 10^{-5}$) and T/T genotype in recessive model ($OR = 1.750$, $P = 0.002$; Table 2). As shown in Table 3, we did not find any other significant differences between MS and HC groups among other previously reported variants within transcription-related genes.

DISCUSSION

MS is an autoimmune demyelinating disease of CNS. Increasing number of evidences hint that MS is strongly associated with genetics. The MHC loci made the early success in demonstrating the important role of genetic factors. To date, 13 MHC loci had been identified.[6] Nevertheless, little progress was made in unraveling the non-MHC genes underlying susceptibility to MS until

Table 3: Other variants involved in transcription regulation between MS and healthy controls

SNP	Location*	Region	Candidate gene
rs228614	Chr4: 102657480	Intronic	*NFKB1/MANBA*
rs11154801	Chr6: 135418217	Intronic	*MYB/AHI1*
rs9321619	Chr6: 137553271	Intergenic	*OLIG3*
rs4410871	Chr8: 127802783	Intronic	*MYC*
rs4902647	Chr14: 68787474	Downstream	*ZFP36L1*
rs2300603	Chr14: 75539214	Intronic	*BATF*
rs744166	Chr17: 42362183	Intronic	*STAT3*

SNP	MS MAF (allele)	HC MAF (allele)	χ^2	P	OR (95% CI)
rs228614	0.48 (A)	0.48 (G)	1.005	0.361	0.864 (0.649–1.150)[†]
rs11154801	0.36 (A)	0.35 (A)	0.067	0.796	1.040 (0.771–1.404)
rs9321619	0.36 (A)	0.39 (A)	0.559	0.455	0.894 (0.665–1.200)
rs4410871	0.30 (T)	0.34 (T)	1.205	0.272	0.842 (0.620–1.144)
rs4902647	0.30 (C)	0.33 (C)	0.648	0.421	0.881 (0.648–1.199)
rs2300603	0.31 (C)	0.27 (C)	2.139	0.144	1.260 (0.924–1.719)
rs744166	–	–	–	–	–

*Position is based on GRCh38.p10 and dbSNP Build 141; †For Allele A. MS: Multiple sclerosis; HC: Healthy controls; SNP: Single-nucleotide polymorphisms; *CI*: Confidence interval; –: Not available; MAF: Minor allele frequency; *OR*: Odds ratio.

the advent of GWAS technology. Over the last decade, over 200 non-MHC genes had been identified using GWAS analyses in large cohorts.[7,8,16] Unfortunately, almost all the GWAS analyses were Caucasian population based. A number of risk loci might not be replicated in Chinese MS patients. In our previous studies, Liu *et al*.[17] found that the variants of interferon regulatory factor 5 were not associated with MS in the southeastern Han Chinese population. Moreover, Cai *et al*.[18] found the association between autophagy-related gene 5 (*ATG5*) and neuromyelitis optica, but failed in MS. Fortunately, Zhuang *et al*.[19,20] identified that the variants in interleukin 7 (*IL7*) and *CYP27B1* were associated with MS. Similarly, in the present study, we confirmed only one previously reported risk variant, rs11129295 in *EOMES* gene. We found that the frequency of T/T genotype was much higher in the MS group and the T allele of rs11129295 increased the risk of MS.

TFs are playing critical roles in the differentiation of Th cell. As was demonstrated previously, T-bet is a major factor for Th1 cell differentiation and IFN-γ production.[21] Similarly, *Foxp3* and *RORγt* are the master TFs of nTreg cell and Th17 cell, respectively.[9,22] *EOMES*, also termed as *TBR2*, encodes a TF which is crucial for embryonic development of the CNS in vertebrates.[23] Besides, multiple lines of evidences have demonstrated that *EOMES* deeply involves in defense against viral infections.[24,25] Its function in CD4+ Th cell differentiation was remarkably noted recently. A functional experiment revealed that *EOMES* expression directly suppresses the *Rorc* and *IL-17a* expressions through binding to the promoter regions of these genes, which results in suppression of Th17 cell differentiation.[26]

Meanwhile, *EOMES* expression itself is suppressed by transforming growth factor beta via a Smad-independent pathway in autoimmune disorders.[26] In addition, a Caucasian population-based mRNA sequencing study further indicated the negative role of *EOMES* dysregulation played in MS progression.[12] However, more recent functional studies were questioning the role of *EOMES* in some specific conditions. One study even revealed that the higher expression of *EOMES* in Th cell could result in the occurrence of secondary-progressive MS.[27] Another study conducted by Lupar *et al*.[28] revealed that the expression of *EOMES* limits the *Foxp3* induction in a cell-intrinsic way. However, more recently, Zhang *et al*.[29] reported that *EOMES* promotes the development of type 1 regulatory T cells, a *Foxp3*-negative, IL-10-producing T cell subset, which has potent immunosuppressive functions in autoimmunity. Thus, the role of *EOMES* playing in the pathogenesis of MS seems rather complicated and might be divided into two phases, acute phase and chronic phase. The more specific mechanisms of *EOMES* in these two phases remain to be clarified further.

In summary, we have identified a transcription regulation-related variant in Chinese MS patients. This variant is associated with increasing risk. The findings in the current study, together with previous studies, enhanced the knowledge that *EOMES* low expression in the acute phase of RRMS could promote the disease progression. As well, it possibly hints that overexpression of *EOMES* in the acute phase of RRMS might be a potential therapeutic target in RRMS.

Acknowledgment

Conflicts of interest

There are no conflicts of interest.

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EOMES基因多态性与中国人群复发缓解型多发性硬化发病风险相关

摘要

背景：多发性硬化（Multiple Sclerosis, MS）是一种常见的中枢神经系统自身免疫性疾病。越来越多的全基因组关联分析（genome-wide association study, GWAS）表明多发性硬化可能与遗传因素密切相关。遗憾的是，几乎所有的GWAS都是基于高加索人群， 实验中得到的很多风险位点并不能在中国MS病人身上得到验证。

方法：自2008年9月2日至2013年6月7日，我们连续收集了142例复发缓解型MS（relapsing-remitting MS, RRMS）患者及301名健康志愿者作为第一阶段的研究对象，通过Sequenom MassArray技术对8个GWAS研究发现的转录调节相关风险位点进行单核苷酸多态性（single-nucleotide polymorphisms, SNPs）分型。为了验证第一阶段所得到的阳性结果，我们自2015年4月7日至2017年6月29日又连续收集了44例RRMS患者及200名健康志愿者，通过Sanger测序进行SNP位点检测。患者与健康志愿者基因型以及等位基因之间的差异、风险比及95%置信区间通过χ^2或Fisher精确概率法进行检验。Hardy-Weinberg平衡亦通过χ^2检验进行计算。

结果：在第一阶段分析中，我们发现仅有*EOMES*基因rs11129295位点这一个风险位点可能与MS发病风险相关，患者中携带T/T基因型的频率显著高于健康志愿者 （χ^2=10.251, *P*=0.005），T等位基因提高MS发病风险（χ^2=10.022, *P*= 0.002）。在第二阶段及联合分析中，rs11129295位点T等位基因依然能提高MS发病风险（χ^2 = 4.586, *P*= 0.030 以及χ^2 =16.378, *P* = 5.19×10^{-5}）。

结论：本研究验证了*EOMES*基因多态位点rs11129295与中国RRMS患者发病风险的相关性，为新的RRMS治疗方法提供了潜在靶点。

Supplementary Table 1: Primers for MassArray

SNP	Candidate gene	PCR primers	MassEXTEND primers
rs228614	*NFKB1/MANBA*	Forward: ACGTTGGATGTGCTTTTACTGTGTTCCTTC	GTCCCATTCAGTGCTTTC
		Reverse: ACGTTGGATGAGTCAGGCTTAAGCAACCAC	
rs744166	*STAT3*	Forward: ACGTTGGATGACATTGAGAGGGCAATTGGG	gggcCTTGAGGGAATCGAGC
		Reverse: ACGTTGGATGTGGCTGTAATGTCTTGAGGG	
rs2300603	*BATF*	Forward: ACGTTGGATGACATAGACTGATGCCGAGAG	cctctTCAGTATGAGGCTTTCATTC
		Reverse: ACGTTGGATGTTCTCTCTAAGCAGCCATCC	
rs4410871	*MYC*	Forward: ACGTTGGATGTCTGCCGTGAATGAGAAACC	CCTCCCACACTGGAA
		Reverse: ACGTTGGATGGCAGTTACATCTGCAGTGTG	
rs4902647	*ZFP36L1*	Forward: ACGTTGGATGTAAGCCTATAGCTCCCTTCC	caCCCGTCCCCTCTAAG
		Reverse: ACGTTGGATGGCTCCTTTGCAGAAAACCTC	
rs9321619	*OLIG3*	Forward: ACGTTGGATGCATCTCTTGTAGTCTGGAGG	CAACTGGGCAGATGG
		Reverse: ACGTTGGATGGGGGCAGGAAGAGCATTAAAG	
rs11129295	*EOMES*	Forward: ACGTTGGATGGCTCATTTAATCTTCACAAC	cctcGGCCAGTTTTCTAACTTCT
		Reverse: ACGTTGGATGGTGACGTGGCCAGTTTTCTA	
rs11154801	*MYB/AHI1*	Forward: ACGTTGGATGAGCTGTCATGTACCATGCAC	ccttaAGAAGGTCGAAACCTCAAGT
		Reverse: ACGTTGGATGCTCCTTCAGAAGGTCGAAAC	

PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphisms.

Supplementary Table 2: Hardy-Weinberg equilibrium tests for all Chinese Han participants in this study

SNP	Candidate gene	MS		HC	
		χ^2	*P*	χ^2	*P*
Stage 1					
rs228614	*NFKB1/MANBA*	1.69	0.19	0.07	0.79
rs744166	*STAT3*	5.95	0.02	2.66	0.10
rs2300603	*BATF*	1.23	0.27	0.62	0.43
rs4410871	*MYC*	1.09	0.30	1.82	0.18
rs4902647	*ZFP36L1*	0.02	0.90	1.42	0.23
rs9321619	*OLIG3*	0.48	0.49	0.01	0.92
rs11129295	*EOMES*	0.77	0.38	0.13	0.72
rs11154801	*MYB/AHI1*	1.72	0.19	0.92	0.34
Stage 2					
rs11129295	*EOMES*	2.91	0.09	2.16	0.14
Stage 1 + 2					
rs11129295	*EOMES*	2.67	0.10	1.64	0.20

MS: Multiple sclerosis; HC: Healthy controls.