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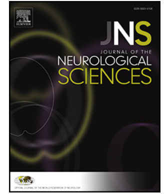

The analysis of correlation between *IL-1B* gene expression and genotyping in multiple sclerosis patients

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ABSTRACT

IL-1B is released by monocytes, astrocytes and brain endothelial cells and seems to be involved in inflammatory reactions of the central nervous system (CNS) in multiple sclerosis (MS). This study aims to evaluate the expression level of *IL-1B* mRNA in peripheral blood mononuclear cells (PBMCs), genotype the rs16944 SNP and find out the role of this SNP on the expression level of *IL-1B* in MS patients. We found that the expression level of *IL-1B* in MS patients increased 3.336 times more than controls in PBMCs but the rs16944 SNP in the promoter region of *IL-1B* did not affect the expression level of this gene and there was not association of this SNP with MS in the examined population. Also, our data did not reveal any correlation between normalized expressions of *IL-1B* gene with age of participants, age of onset, and disease duration.

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1. Introduction

Multiple sclerosis (MS) is an autoimmune/inflammatory disease of the central nervous system (CNS) with an unknown etiology [1]. Much progress has been made towards understanding the role of genetic factors in MS pathogenesis, but the only consistent association with MS to date, is the extended haplotypes of the major histocompatibility complex (MHC), particularly those containing the HLA-DRB1*1501 allele [2]. The contribution of genes encoding cytokines is currently under elaborate investigation, as cytokines are important mediators in immune and inflammatory disease [3]. IL-1a and IL-1B are pro-inflammatory cytokines with pleiotropic activities including differentiation and growth of T- and B-cells [4]. IL-1B is released by monocytes, microglial cells, astrocytes and brain endothelial cells and seems to be involved in inflammatory reactions of the CNS [5]. IL-1 is present in and around MS lesions, and might be involved in the destruction of CNS myelin [6]. Also, the concentration of IL-1 in the cerebrospinal fluid (CSF) of MS patients is correlated with disease activity [7]. Specific genotypes of IL-1 or the balance between IL-1 and its receptor antagonist (IL-1Ra), are known to be associated with clinical severity and/or progression of the disease [8]. There are some evidences that the expression of some genes *in cis,* may be affected by some non-coding SNPs which confers susceptibility to disease development [9,10]. One polymorphism with functional properties has been described for *IL-1B* gene, at the position of −511 (rs16944) in the promoter region [11]. Moreover measurements of cytokine expression levels and their correlation with some clinical features of patients often provide additional pathological information. Also, complete molecular mechanisms of cytokine signaling pathways provide new ways in the development of new drugs. Here, we hypothesized that the *IL-1B* as a critical factor in the destruction of the blood brain barrier (BBB), overexpressed in peripheral blood mononuclear cells (PBMCs) of MS patients and rs16944 SNP on the promoter of this gene may affect its expression. There were no previous studies which showed the abnormal expression of *IL-1B* in PBMCs of MS patients. Hence in this study, the systemic expression of *IL-1B* in PBMCs, the association of rs16944 SNP with MS and the effect of this SNP on the expression level of *IL-1B* were investigated. Moreover, we analyzed the correlation between normalized expression of *IL-1B* with age of participants, age of onset, and disease duration.

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2. Materials and methods

2.1. Patients and controls

In this study, to evaluate the expression of *IL-1B,* we selected 30 MS patients and 30 age and gender matched healthy controls, all from Sistan and Baluchistan, the southeast province of Iran. All patients were taking Cinnovex which is an interferon-beta based drug, as a general treatment strategy in a weekly period. To optimize the role of this treatment in the expression of *IL-1B*, the blood samples were collected after a week of injection and just before the new injection. Genotyping assessed 114 MS patients and 127 age and gender matched controls including those who participated in expression analysis. All patients are chosen according to McDonald criteria [12] and MRI test was performed for all. The informed consent was obtained from all of participants and this study was approved by the Ethics Committee of the University of Zabol.

2.2. IL-1B expression

The whole blood was collected in 5 ml EDTA tubes from 30 MS patients and 30 healthy controls. PBMCs were isolated by density gradient centrifuge using Ficoll/Paque solution (Sigma Chemical Co., St. Louis, MO; New York, NY). Total RNA was extracted using TRIzol reagent (IsoGene Lab, Moscow, Russia) according to Chomczynski protocol [13]. RNA concentration, quality and integrity were verified by spectrophotometry (Eppendorf BioPhotometer plus, Eppendorf, Germany) and 1% agarose gel electrophoresis. cDNA was synthesized according to the manufacturer's instructions of Viva 2-Steps RT-PCR kit (Vivantis Technologies, Selangor, Malaysia) with random and OligodT primers. Real time PCR performed by Applied Biosystems 7500 Real Time PCR System (Applied Biosystem/MDS SCIEX, Foster City, CA, USA). The relative expression of the *IL-1B* gene (forward: AGGGACAGGATATGGAGC AACAAG & reverse: CATCTTTCAACACGCAGG-ACAGGT) was normalized to *GAPDH* (forward: CCATGAGAAGTATGACAAC & reverse: GAGTCC TTCCACGATACC) which was selected as the reference gene. The specificity of real time PCR reaction was verified by a single band after gel electrophoresis.

2.3. Genotyping of IL-1B

Blood samples were collected into 5 ml EDTA tubes and genomic DNA was extracted from peripheral blood leukocytes using the boiling method and stored in −20 °C for future analysis. The sequence of rs16944 SNP was obtained from the SNP database (available at http://www.ncbi.nlm.nih.gov). The promoter region containing the *Ava*I polymorphic site of the *IL-1B* gene was amplified by PCR (forward: 5′-TGGC ATTGATCTGGTTCATC-3′, reverse: 5′-GTTTAGGAATCTTCCCACTTAC-3′). The PCR products were digested with 5 units of *Ava*I restriction enzyme (Takara Bio Inc, Japan) at 37° for 16 h according to the manufacturer's instructions. Digested fragments were analyzed after electrophoresis on 12% PAGE and stained with ethidium bromide.

2.4. Statistical analysis

The statistical differences in the expression level of *IL-1B* and the fold changes in patients and controls were compared via independent *t*-test and ΔΔCt methods, respectively. Moreover, we used the chi-square test with a threshold of 0.05 to see whether the alleles are in Hardy–Weinberg equilibrium (HWE). Also, genotypic and allelic frequencies were compared by the chi-square test for cases and controls to analyze the association of this SNP with MS. Genotypes were divided into three groups including: major allele homozygous, heterozygous and minor allele homozygous variants and for each genotype, 95% CI (confidence interval) and OR (odds ratio) were calculated. The statistical *P*-level of significance was also evaluated after Bonferroni's correction for multiple testing. Statistical analysis was performed using SPSS software (Version 20; SPSS Inc, Chicago, IL). Moreover, the correlation between gene expression levels and genotypes was assessed by two-sided Mann–Whitney *U*-test using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Finally, the correlation between normalized expression of *IL-1B* gene with age of participants, age of onset and disease duration was assessed by Pearson's correlation coefficient.

3. Results

3.1. Patients and controls

The patients who resided in Sistan and Baluchistan province for generations, aged between 19 and 55 years and only treated with Cinnovex were included while the patients treated with other drugs, patients who migrate from other provinces, patients who were hospitalized in the time of study, pregnant females and any patient and control who had any inflammation situation were excluded. Patients and controls were matched by age, gender and time of blood sampling. The mean age of disease onset was 27.26 (ranging from 17 to 46) years, and the mean duration of disease was 5.46 (ranging from 1 to 31) years. Some clinical features of patients and controls were presented in Table 1.

3.2. IL-1B expression

The expression analysis of *IL-1B* gene was measured as Ct (cycle threshold) and ΔCt values (Fig. 1). After the statistical analysis, our results revealed that, *IL-1B* was significantly (3.336 times) higher expressed in the PBMCs of MS patients versus healthy controls ($p = 0.005$). The chosen significance level was $p < 0.05$.

3.3. Genotyping

All patients and controls were in HWE for rs16944 SNP. After a Bonferroni correction, the significance level was $p \leq 0.05$. The analysis of allele and the genotype frequencies of rs16944 SNP revealed no significant statistical differences in patients in comparison with normal groups ($P = 0.447$, OR: 1.175, 95% CI = 0.673–2.050) (Table 2). The stratification for gender revealed that, there was no association between rs16944 SNP and MS susceptibility in male and female groups (Table 3). In addition, in stratification for disease progression in MS subtypes, we did not find any significant association with MS, but this association in SP-MS patients showed tendency to be significant ($P = 0.08$, OR: 1.99, 95% CI = 1.121–3.534) (Table 4).

Table 1
Demographic and clinical features of MS patients and healthy controls.

	MS patients				Controls	
	Number	Male/female (%)	RR/SP/PP (%)	Age of onset (average ± sd)	Number	Male/female (%)
rs16944 SNP Genotyping	114	32.5/67.5	64.9/23.7/11.4	29.5 ± 6.45	127	44.1/55.9
IL-1B expression	30	26.7/73.3	100/0.0/0.0	27.26 ± 7.14	30	20/80

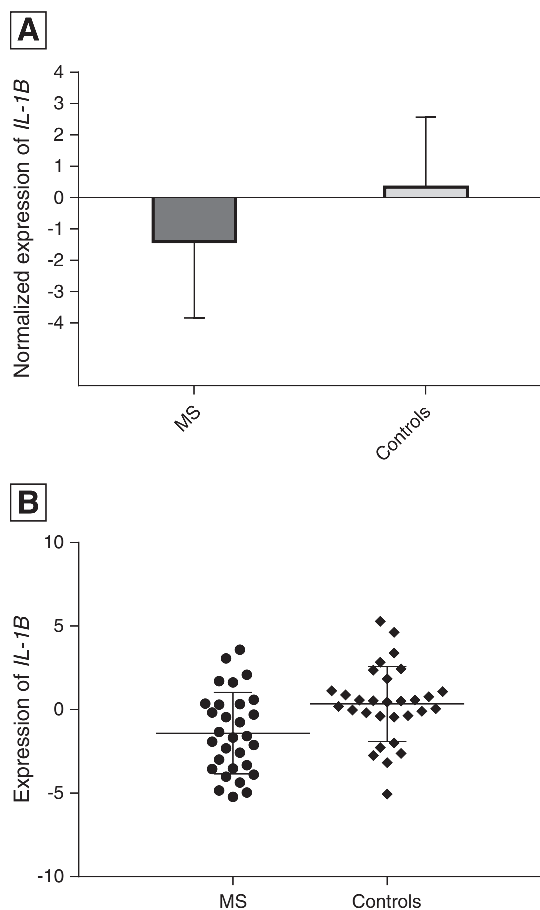

Fig. 1. The expression analysis of *IL-1B* gene. *IL-1B* was significantly (3.336 times) higher expressed in the PBMCs of MS patients versus healthy controls ($p = 0.005$). A) Normalized expression of *IL-1B* and B) Ct values of patients and controls.

3.4. The effect of rs16944 SNP on IL-1B expression

We consistently grouped homozygous individuals for the *IL-1B* T putative risk allele together with those that are heterozygous for this allele, and they were compared with the individuals being homozygous for the protective C allele. We also performed this analysis in MS and control groups only in females. Our analysis revealed that, the presence of rs16944 T allele in *IL-1B* gene did not affect the expression of *IL-1B* mRNA in PBMCs of MS patients ($P = 0.421$), controls ($P = 0.2$) and female groups ($p = 0.1$ and $p = 0.63$) (Fig. 2). We could not perform this analysis in the male group due to the low number of cases in MS and control groups of expression study.

3.5. Correlation between normalized expression of IL-1B with age of participants, age of onset, and disease duration

To find a possible relation between the expression of *IL-1B* gene with age of participants, age of onset and disease duration, a correlation study was done. Our results did not reveal any correlation between normalized expression of *IL-1B* and age of participants. Also we did not find any correlation between normalized expression of *IL-1B,* age of onset and disease duration in total and in gender stratification (Table 5).

4. Discussion

IL-1B may contribute to glial activation, expression of inflammatory molecules, changes of blood brain–barrier permeability, apoptosis and/or necrosis of neurons and oligo-dendrocytes, as well as invasion of immune cells into the CNS [14–17]. For the first time based on the best of our knowledge and in opposite to Huang's study [18], we have shown that the expression of *IL-1B* in PBMCs of MS patients was 3.336 times higher than healthy controls while the normalized expression of *IL-1B* did not show any correlation with the age of the participants, disease duration and age of onset. Also our analysis did not significantly show different genotype-dependent effects of rs16944 SNP on *IL-1B* expression in PBMCs of the patients in comparison to healthy controls and in female group. This result indicated that the gene expression level was characteristic for individuals and may be useful for personalizing therapy. The contribution of a common genetic polymorphism to disease severity may be independent from its effect on susceptibility. It has been previously shown that, the polymorphisms in *IL-1* family genes affect the transcriptional activities and MS severity [4,19]. In agreement with Leikfoss et al. [20] whole-blood samples are heterogeneous in their cellular composition and may influence our results.

Modulating the expression of *IL-1B* might be useful in combinational therapies and in assessing the positive responses to immunomudulating therapies. Besides the rare presence of IL-1B in the normal CNS tissues, increased expression of *IL-1B* has previously been reported in sera, CNS and brain lesions of MS patients due to MS pathogenesis [21,22]. Our results and other evidences help this hypothesis that the cells which

Table 2
The information of rs16944 SNP of *IL-1B* gene. We did not find any significant association of rs16944 SNP with susceptibility to MS in studied population.

Gene	SNP	Chromosome position	Major/minor allele	Risk allele	Cases risk allele (%)	Controls risk allele (%)	OR (95% CI)	*P*-value
IL-1B	Rs16944	Chr2; promoter region (−511)	C/T	T	43.8	48.5	1.175 (0.673–2.050)	0.447

Table 3
Genotyping analysis with stratification for gender of MS patients. In male and female groups no significant association was found between rs16944 SNP and MS.

SNP	Group	Female risk allele (%)	OR (95% CI)	*P*-value	Male risk allele (%)	OR (95% CI)	*P*-value
Rs16944	MS	46.1	1.128	0.326	39.1	1.387	0.511
	Controls	49.2	(0.647–1.965)		47.3	(0.791–2.433)	

Table 4
Stratification of MS patients by subtypes. No association was found between rs16944 SNP and MS progression in different subtypes of MS.

Patient subtypes	RR risk allele (%)	OR (95% CI)	*P*-value	SP risk allele (%)	OR (95% CI)	*P*-value	PP risk allele (%)	OR (95% CI)	*P*-value
Rs16944	49	0.961 (0.552–1.673)	0.955	31.5	1.99 (1.121–3.534)	0.080	42.3	1.275 (0.729–2.228)	0.233

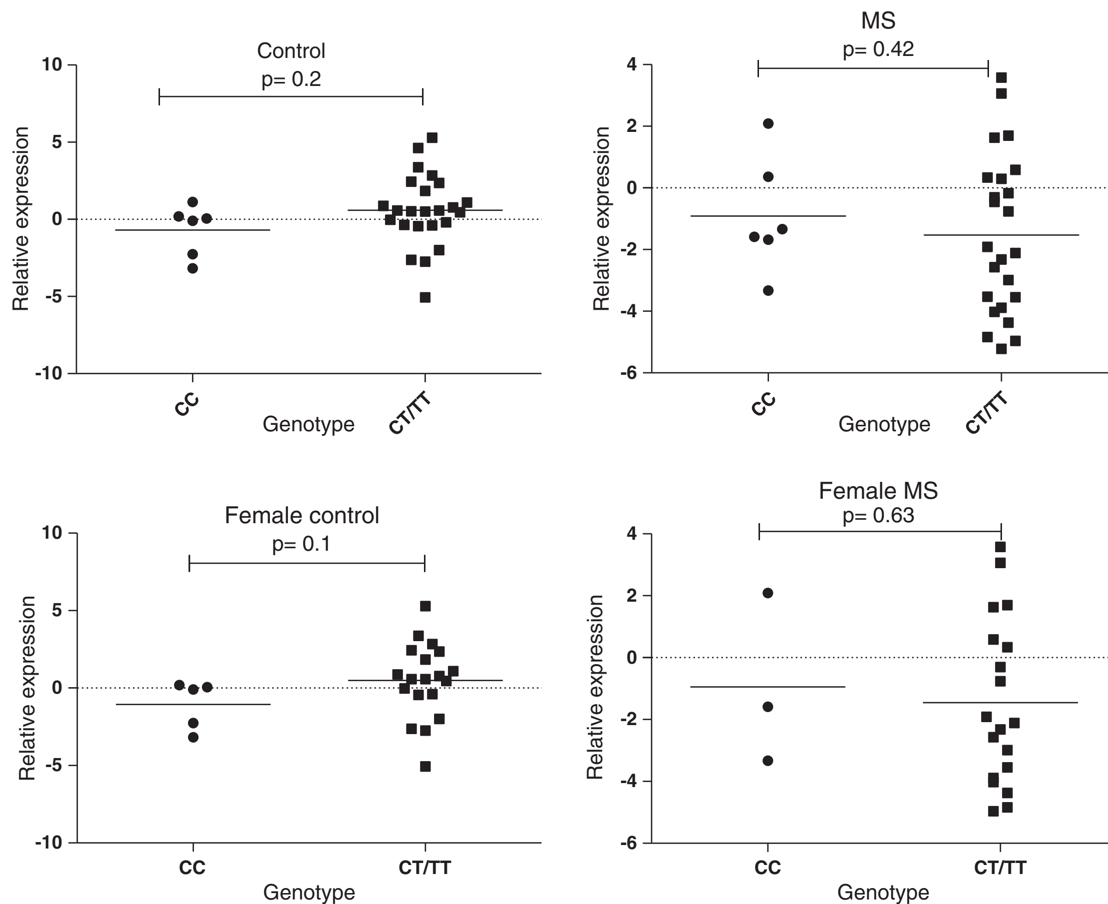

Fig. 2. The effect of rs16944 SNP on *IL-1B* mRNA expression. The graphs show relative expression of *IL-1B* normalized to *GAPDH* in PBMCs of 30 MS patients and 30 healthy controls (up) and also in stratification for female group (down), versus to genotype of rs16944 T SNP. Individuals carrying the putative risk allele were compared with individuals homozygous for the protective allele. No significant association was found for correlation between gene expression levels and genotypes in the MS group.

reside in whole blood are triggers for inflammation responses in the brain plaques, as the expression of *IL-1B* increases firstly in the blood cells, destroys the blood–brain barrier and finally, it can be seen in the plaques. Here we suggest that the increased expression of *IL-1B* could serve as a possible biomarker for MS diagnosis.

There are some SNPs in the *IL-1B* gene which were investigated in the association studies of MS in different populations [2,8,23]. Among them, we selected rs16944 SNP which is located in the promoter region of *IL-1B* and we can analyze its effect on the expression of *IL-1B* in PBMCs. Our results in agreement with two other studies in Iranian population [24,25] did not show any significant association of rs16944 SNP and MS susceptibility, but we did find a tendency for association in SP-MS patients, that might be significant in a larger group of samples. This SNP did not show any association with MS in different populations [26–28], but former studies showed a decreased risk for developing MS in carriers of IL-1B (−511)*T allele [2,29] and later occurrences of MS with faster disability progression in IL1B −511 TT genotype reported in other studies [30]. In combination, these data showed that, the rs16944 SNP may produce a very low susceptibility for MS at least in Iranian population or in Sistan and Baluchistan racial group. But other studies with larger sample sizes are needed to confirm the exact association of *IL-1B* gene with MS in this population. It is notable that, Sistan and Baluchistan province has a special climate and a unique genetic background due to high rate of consanguineous marriage [31]. The number of patients registered in the medical network of Sistan and Baluchistan that have our inclusion criteria is restricted. Also, we were not able to access the EDSS score for all patients and therefore, we did not perform the correlation of *IL-1B* expression and EDSS. We suggest this analysis to be replicated in a larger number of samples and the effect of rs16944 SNP on the expression levels of *IL-1B* to be analyzed in more homogenous cell lines. We hope that our results encourage the researchers for further investigation.

Table 5
Correlation between normalized expressions of *IL-1B* gene, age and disease duration. We did not find any significant correlation between the expression levels of *IL-1B* gene, age of participants, age of onset, and disease duration.

Correlation	*r*	*P* value
MS–age	0.073	0.69
MS–onset	0.048	0.79
MS–duration	0.002	0.79
Control–age	−0.006	0.97
Female MS–age	0.007	0.97
Female MS–onset	0.078	0.72
Female MS–duration	−0.09	0.68
Female control–age	0.08	0.7
Male MS–age	0.34	0.39
Male MS–onset	0.01	0.97
Male MS–duration	0.32	0.42
Male control–age	−0.35	0.49

Conflicts of interest

The authors have no conflicting financial interests.

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