BRIEF COMMUNICATION
Iran J Allergy Asthma Immunol
February 2016; 15(1):75-81.

Quantitative Evaluation of BAFF, HMGB1, TLR 4 and TLR 7 Expression in Patients with Relapsing Remitting Multiple Sclerosis

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Received: 15 March 2015; Received in revised form: 4 April 2015; Accepted: 10 May 2015

ABSTRACT

Multiple sclerosis is a chronic inflammatory disease of the central nervous system characterized by a complex immune response. Because of the complex nature of MS pathogenesis, a panel of biomarkers derived from different platforms will be required to reflect disease-related alterations.

Monitoring and evaluation of molecules associated with the pathogenesis of the disease would provide useful information on disease progression and therapeutic assessment. In view of this, we evaluated the mRNA expression levels of B-cell activating factor (BAFF), high mobility group box 1 (HMGB-1), Toll like receptor (TLR) 4 and TLR7 in MS. These molecules are implicated in the pathogenesis of MS; however, they have received little attention. PBMCs were isolated from whole blood of 84 relapsing remitting multiple sclerosis patients and 70 healthy controls. Relative quantitative RT-PCR was applied to quantify the transcriptional levels of the immune markers.

The mRNA expression levels of TLR7 were significantly elevated in RRMS patients than healthy controls. TLR4 expression was found to be significantly lower in the patients than control group. We found no difference analyzing the mRNA levels of BAFF and HMGB1. Our data highlights the immune marker correlates in RRMS patients.

However, further in-depth studies are warranted to check the role and the relevance of these immune markers in autoimmune diseases such as MS.

Keywords: Biomarkers, Cytokines; HMGB proteins; Inflammation; Multiple sclerosis; RNA messenger

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Multiple sclerosis (MS) is a neurological disease caused by the malfunction of the immune system, leading to an autoimmune response against antigens of cerebral white matter tissue.[1] The pathologically distinguishing features of the disease are demyelination, axonal loss, and inflammation.[2] The MS disease affects around 2.5 million people worldwide [3] and commonly found in young adults, and it is more common among women.[4] The prevalence of MS in Iran is generally considered to be relatively high,[5, 6] in which Isfahan and Golestan cities are supposed to have the highest and the lowest prevalence, respectively.[6, 7] Indeed, a study performed in 2010 by Isfahan MS society (IMMS), reported a prevalence of 73.3/100,000 between April 2003 and July 2010.[8] The relapsing-remitting form of MS (RRMS) was considered as the most common type.[9]

The major clinical symptoms of MS are cognitive disabilities,[10] abnormal sensation, paralysis, and ocular symptoms associated with relapses and remissions.[11] However, the symptoms vary depending on which part of the central nervous system (CNS) is involved.[10] Although the etiology of MS is not fully understood [12], there is evidence of the interplay between genetic susceptibility and environmental factors.[13, 14] Indeed, studies investigating the genetic components have highlighted a role for genetic in this polygenic disease.[3]

Humoral and cellular components of the immune system were shown to be involved in the development of autoimmune diseases such as MS.[15] This notion could be supported by the fact that T-cells, macrophages, immune mediators such as cytokines were found in active MS lesions.[15] The characteristic features of RRMS form indicates the effects of pro- and anti-inflammatory responses occurring over the time.[11] Indeed, the progression of the disease in MS patients was associated with an increase in proinflammatory and down-regulatory cytokines because inflammatory and restorative processes were suggested to occur concurrently.[11] Analysis of proinflammatory cytokines is useful for assessment of cytokines profile in MS patients and healthy individuals.[16]

Few years back, all attempts to find acceptable immune markers for MS remained largely unsuccessful, due to the multifactorial nature of the disease.[17] Interestingly, existing knowledge in the field of genomics and gene expression has provided a number of immune markers for assessment of the disease.[17] Indeed, the dys-regulation of several immune markers is suggested to be important in facilitating the MS development.[18] BAFF, HMGB-1, TLR4 and TLR7 are among immune markers which have received little attention in MS. BAFF is an efficient molecule regulating the development and function of B cells.[19] Abnormal high production of antibody is associated with over expression of BAFF, results in some autoimmune diseases, which rely on innate immune mechanisms without involvement of T cells [20]. Furthermore, BAFF has been produced by macrophages, dendritic cells, and/or neutrophils and these cells could be recruited to inflammatory site.[21, 22] HMGB-1 is secreted by macrophages, monocytes and dendritic cells as a cytokines mediator of inflammation [23] and its interaction with TLR4 results in production of cytokines.[24] Similarly, TLR7 was shown to be important in the development of effective immunity.[25] These molecules can directly or indirectly involve in inflammatory responses; therefore, monitoring the expression levels of these molecules could be informative for the prediction of disease progression and treatment decision for prevention and control of the disease.

In this study, we applied relative quantitative real time PCR technique to evaluate the transcriptional levels of BAFF, HMGB-1, TLR4 and TLR7 in RRMS patients and healthy subjects.

MATERIALS AND METHODS

Patient Characteristics

Eighty four RRMS patients (24 male, 60 female; mean age 34.55 +/- 8.83 years) attending Sina Teaching Hospital in Tehran and diagnosed according to McDonald criteria were recruited.[26] In addition, 70 healthy control subjects (20 male, 50 female, mean age 34.16 +/- 8.26 years) were also included (Table 1). All RRMS patients, except eight, were receiving treatment. All participants had given informed consent to provide 5ml blood samples for the study, which was approved by the Local Research Ethics Committee of Tehran University of Medical Sciences (approval No: 92-01-27-21999).

PBMCs Isolation from Whole Blood

Blood samples were obtained from patients and healthy volunteers and transported to the laboratory. PBMCs were isolated from fresh whole blood containing preservative-free heparin by using Ficoll-Paque centrifugation (GE Healthcare, Amersham, UK) and resuspended in RPMI 1640 medium (Gibco BRL Life Technologies, USA) supplemented with 10% heat-inactivated FCS (Gibco). The isolated PBMCs were counted and stored in liquid nitrogen until use for RNA extractions.

RNA Extraction and cDNA Synthesis

The extraction of RNA from PBMCs was carried out using TRIzol reagent according to the manufactures' protocol (Invitrogen, Carlsbad, CA). The extracted RNA was first eluted in 50 µl of RNase-free water, and then treated with RNase-Free DNase I (Invitrogen) according to the manufacturers to eliminate contaminating genomic DNA. The concentrations were measured by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at a wavelength of 260 nm. The pure RNA was adjusted to a final concentration of 1 µg and cDNA was synthesized from total RNA (1 µg) using reverse transcriptase AMV kit (Roche, Berlin, Germany), and for single cycle in a final volume of 20µl as follows: 10 min at 22°C, 45 min at 45°C for RT, and 5 min at 95°C then stored in -80^{0}C until use.

Determination of mRNAs Levels of Immune Markers

Relative quantitative (RQ) RT-PCR was applied to quantify the mRNAs levels of BAFF, HMBG-1, TLR 4 and 7 using SYBR green Real-Time PCR master mix (Invitrogen) on the ABI step one plus (Applied biosystem, Carlsbad, CA, USA). Briefly, PCR amplification reactions were carried out in 20µl reaction mixtures containing cDNA (10 fold diluted), 2X SYBR Green supermix and 10 pmol of each primers (Table 2). The reactions were incubated at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15s, 57°C for 30s and 68°C for 30s. A melting curve analysis was performed to confirm single gene-specific peaks. The linearity and accuracy of Real time RT-PCR was evaluated using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Reference gene). The expression was analyzed according to the $2^{-\Delta\Delta CT}$ method. The relative values of each biomarker were expressed as change fold of the MS group over the control group to compare mRNA levels between subjects.

Table 1. Demographic characteristic of RRMS patients and Healthy controls

Subjects	Gender		Age (years)
	Male	Female	
RRMS Patients[#] (N=84)	[*]24 (32; 19-54)	60 (35; 20-49)	32 (26; 19-30) 29 (35; 31-39) 23 (44.5; 41-54)
Healthy controls (N=70)	20 (34; 22-53)	50 (32; 16-50)	28 (27; 16-30) 25 (36; 31-39) 17 (46; 41-53)

[#]Eight (out of 84) RRMS patients were drug naïve. [*]Represents number of subjects (median age (years); and age range)

Table 2. Primer sequences of the four immune markers and GAPDH genes

Genes	Forward	Reverse
BAFF	5´-GGCCCCAACCTTCAAAGTTC-3´	5´-GCGTGACTGCTCCCTTTCTG-3´
HMGB1	5´-TGGCTCCCGCTCTCACA-3´	5´-CCG GCG CTG TCT CTA TGG-3´
TLR4	5´-TGGTGTCCCAGCACTTCATC-3´	5´-GCCAGGTCTGAGCAATCTCAT A-3´
TLR7	5´-TCTCGAGGAAAGGGACTGGTT-3´	5´-GCTAAGCTGTATGCTCTGGGAAA-3´
GAPDH	5´-ATGGAAATCCCATCACCATCTT-3´	5´-CGCCCCACTTGATTTTGG-3´

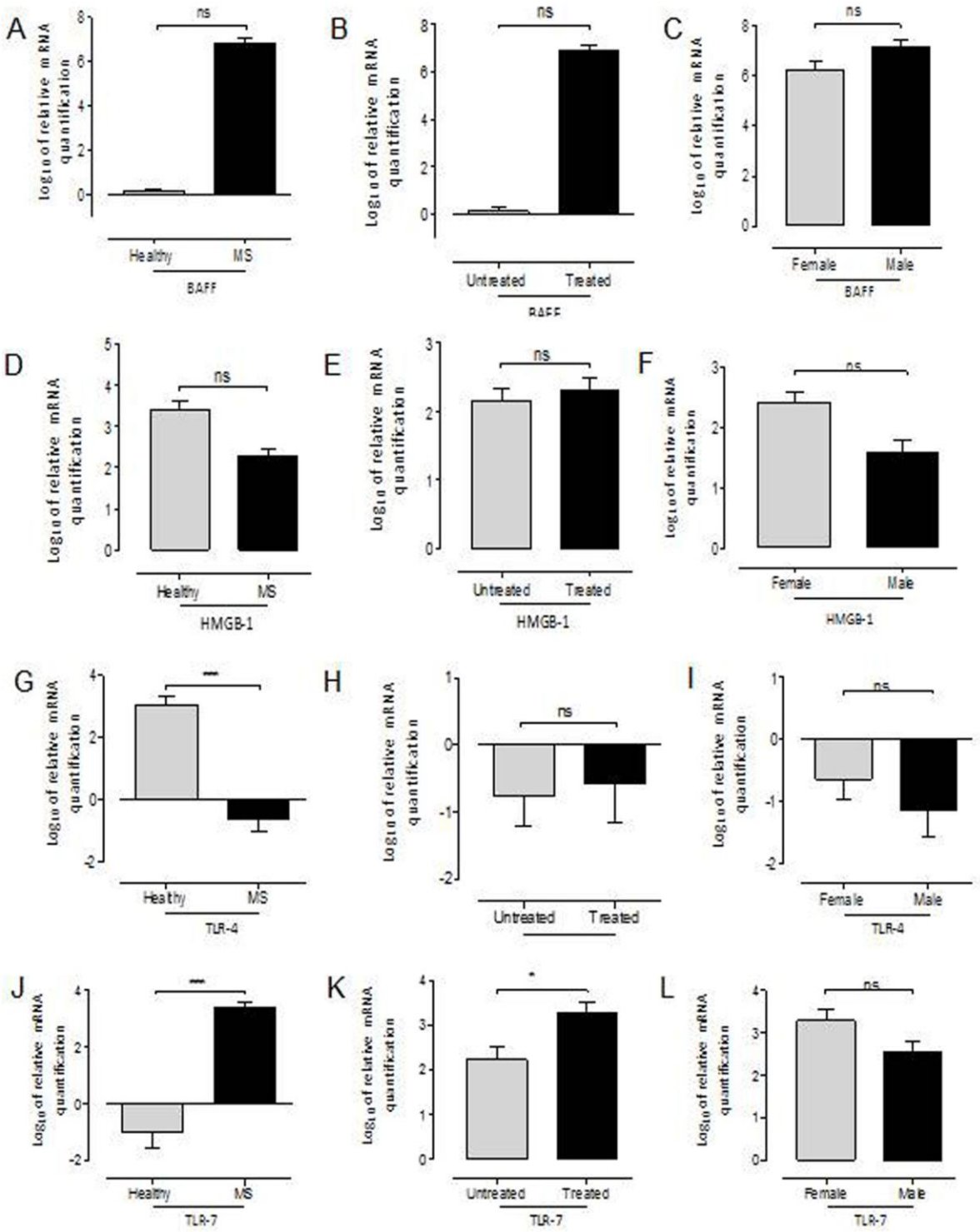

Figure 1. Real-time PCR were used to quantify the expression of some immune markers on PBMCs from RRMS patients and Healthy controls (left column). Data obtained from RRMS patients were further analyzed based on treatment received and gender (middle and right columns, respectively). (A-C) Quantification expressed as log10 of BAFF expression, (D-F) HMBG-1expression, (G-I) TLR4 expression and (J-L) TLR7 expression. Bars indicate mean percentage values and *p*-values were calculated with Mann Whitney test.

Statistical Analysis

GraphPad Prism software (GraphPad Software, Inc., La Jolla, California) was used for plotting graphs and for statistical analysis. The Mann-Whitney test and Student's t test were used accordingly, and $p \leq 0.05$ were considered as significant

RESULTS

Demographic Description of the Subjects

Demographic features of RRMS patients and healthy control are shown in Table 1. As indicated, the sex ratio of the RRMS group was 2.5:1, and a similar age distribution was observed between male and female. Accordingly, the sex ratio and the age distribution for healthy group were similar to RRMS patients.

Quantification of mRNA Levels

Total RNA was extracted from PBMCs and reverse transcribed into cDNA in accordance with the manufacture's protocol. To perform RQ real-time PCR by the comparative CT method, the CT for the target genes (BAFF, HMGB-1, TLR4 and TLR7) and the reference gene (GAPDH) were determined in RRMS patients and healthy controls.

The expression level of TLR4 was found to be significantly lower in RRMS patients when compared with healthy controls as indicated in Figure 1G (MS: 0.239±0.147 vs healthy: 1177±965; $p<0.0001$). Moreover, the level of mRNA for TLR7 was significantly higher in RRMS patients than healthy group (RRMS: 2349±1501 vs healthy: 0.0934±0.0651; $p<0.0001$) (Figure 1J). However, we could not find any significant difference between RRMS patients and healthy group for HMGB-1 and BAFF ($p>0.05$).

As shown in Figures 1B, 1E, and 1H, no significant differences were observed for BAFF, HMGB-1, and TLR4 ($p>0.05$) when data further analyzed based on treatment received, except for TLR7 where the mRNA expression level was found to be higher in treated RRMS patients than untreated ones (treated 1828±1518 vs untreated 174±163; $p=0.0142$) (Figure 1K). In addition, the differences observed between male and female were not statistically significant (Figures 1C, 1F, 1I and 1L).

DISCUSSION

Although previous studies have recognized inflammatory immune responses in MS, however the exact pathogenesis of the disease is not well understood. Immune dysregulations are implicated in MS pathogenesis.[27] Indeed, TLRs are known to trigger an innate immune response, and TLR4 is considerably important in the initiation and exacerbation of inflammatory disease.[27]

Looking at the transcriptional levels of BAFF, we found no significant difference between RRMS patients and healthy group although the numerical values showed difference. Our result is not in agreement with previous study which reported higher expression of BAFF mRNA in MS patients than in healthy controls.[28] Our finding suggests normal regulation of B cell immunity in the MS patients; however, exposure to microbial antigens has been shown to up-regulate the BAFF production.[29]

Similarly, we found no significant difference in the mRNA expression level of HMGB-1, suggesting that HMGB-1 expression level may not be indicative of MS exacerbation. However, it has been suggested that the expression of HMGB-1 in activated macrophages and resident microglia may intensify the inflammatory process in MS and EAE.[30]

TLRs in innate immune system are reported to mediate important signals for the activation of cells of the adaptive immune system, including autoreactive lymphocytes.[24] As shown, the expression level of TLR4 was significantly lower in RRMS patients compared to healthy subjects. TLRs are key components of the innate immune system and the lower expression of TLR4 in the MS patients may represent the condition of innate immune activation.[25] Moreover, it has been documented that TLR4 expression on different immune cell types such as lymphocytes,[31] can specifically recognize bacterial lipopolysaccharide, endogenous molecules produced during tissue damage,[32] and HMGB-1.[31]

Unlike TLR4, the mRNA level of TLR7 was significantly higher in RRMS patients than control group. TLR7 expression was also found to be higher in treated patients than untreated ones. Indeed, treatment with interferon-β has been shown to upregulate the expression of TLR7 on PBMCs of MS patients.[25] The majority of the RRMS patients enrolled in this study

received interferon treatment.

However, no significant differences were found when data from RRMS patients analyzed based on treatment received for BAFF, HMGB-1, and TLR4 immune markers. Our data indicate that there is no correlation between the treatment and the expression level of these immune markers in the RRMS patients. However, to further analyze the significance of these molecules, larger group of MS patients with and without history of medication should be included.

Looking at gender, we found no significant difference in the mRNA expression levels of male and female RRMS patients, suggesting that the gender may not necessarily be a determinant factor for the transcriptional levels of these immune markers in MS patients. A limitation of this study was that we were unable to analyze the protein levels of these immune markers and their corresponding cytokines.

In general, TLR4 and TLR7 expression highlight the importance of these molecules although further in-depth studies are warranted regarding their role and relevance in MS.

ACKNOWLEDGEMENTS

This work was supported by Tehran University of Medical Sciences, International Campus (TUMS-IC) [Grant Number 92-03-103-23671]

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