ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2024; 23(2):182-196.
DOI: 10.18502/ijaai.v23i2.15324

Altered Expression of B Cell Receptor Signaling Pathway Genes in Peripheral Blood of Patients with Multiple Sclerosis

Shirin Jalili[1], Hadi Shirzad[2], and Seyed Amin Mousavi Nezhad[2]

[1] Institute of Police Equipment and Technologies, Policing Sciences and Social Studies Research Institute, Tehran, Iran
[2] Research Center for Life & Health Sciences & Biotechnology of the Police, Directorate of Health, Rescue & Treatment, Police Headquarter, Tehran, Iran

Received: 16 October 2023; Received in revised form: 31 January 2024; Accepted: 9 February 2024

ABSTRACT

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease and has adverse implications. The exact mechanism of its pathogenesis is not fully understood and remains to be elucidated. In the current study we aimed to identify key genes that can serve as potential biomarkers and therapeutic targets for MS and shed light on pathogenesis mechanisms involved in MS.

We analyzed a gene expression dataset (GES21942) and found 266 differentially expressed genes (DEGs) including 183 upregulated and 83 downregulated genes in MS patients compared to controls. Then we conducted pathway enrichment on DEGs and selected the top enriched pathway i.e., B cell receptor signaling pathway, and 5 genes of this pathway (*CR2*, *BLK*, *BLNK*, *RASGRP3,* and *KRAS*) for further investigation in our clinical samples. We recruited 50 MS patients and 50 controls and assessed the expression of selected genes in the circulation of patients versus controls.

Expression of *CR2*, *BLK*, *BLNK,* and *RASGRP3* were significantly higher in MS cases compared with controls. There was no significant difference in expression of *KRAS* between patients and controls. All of the selected genes with differential expression had noticeable diagnostic power and *CR2* was the most robust gene in differentiating MS cases from controls. Additionally, a combination of genes resulted in enhanced diagnostic power.

Collectively our results suggest that the B cell receptor signaling pathway and the selected genes from this pathway may be implicated in the pathogenesis of MS and each of these genes can be considered as potential diagnostic biomarkers and therapeutic targets.

Keywords: B cell; B cell receptor signaling pathway; Gene expression; Multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory and autoimmune neurodegenerative disease that affects the central nervous system (CNS) and is the main cause of nontraumatic disability among young adults.[1] MS prevalence has increased globally since 2013. Approximately there are 2.8 million patients with MS in

Corresponding Authors: Shirin Jalili, PhD;
Institute of Police Equipment and Technologies, Policing Sciences and Social Studies Research Institute, Tehran, Iran. Tel: (+98 21) 8188 6062, E-mail: jalili.shirin@yahoo.com

Hadi Shirzad, MD, PhD;
Research Center for Life and Health Sciences and Biotechnology of the Police, Directorate of Health, Rescue and Treatment, Police Headquarter, Tehran, Iran. Tel: (+98 21) 8188 6062, E-mail: hadi_shirzad@yahoo.com

the world and its prevalence rate is 35.9 per 100,000 population on a worldwide basis. The highest prevalence rate has been observed in Europe and North America. The overall incidence rate of MS is estimated to be 2.1 per 100,000 persons/year across 75 reporting countries and the average diagnosis age is 32 years old.[2] Iran has the highest prevalence rate relative to other Asian countries with an age-standardized prevalence of 137.6 per 100,000 people.[3]

As an autoimmune demyelinating disease, MS leads to demyelination of neurons and variable levels of axonal damage that cause the development of large focal lesions in the CNS. Involvement of different areas of the CNS such as sensory, visual, motor, and autonomic systems results in heterogeneity in signs and symptoms of the disease.[4] MS is a complex and multifactorial disease where both genetic and environmental factors such as Epstein–Barr virus (EBV) infection, vitamin D, smoking, etc. affect the MS risk.[5] Studies have shown that first-degree relatives of MS patients have a greater susceptibility to MS than the general population. For instance, the risk of MS in children of families where both parents have MS is 10 times greater than that of the general population.[6] So far, several genetic variants have been discovered that show an association with MS susceptibility, such as HLA-DRB1*15:01 which is strongly associated with MS susceptibility.[7]

The exact cause and cellular and molecular mechanisms of MS are not known, despite significant advances in our knowledge of MS pathophysiology and devising novel therapies for the treatment of MS.[8] MS has traditionally been categorized as an autoimmune disease mediated by T cells. Additionally, numerous studies have consistently documented the presence of T cells in CNS lesions of individuals with MS.[9] However, the success of therapies targeting B cells poses a challenge to the traditional belief that T cells are primarily responsible for this autoimmune disease.[10] B cells can contribute to the pathogenesis of MS through different pathways such as antigen presentation to autoreactive T cells, inflammatory cytokines secretion, and autoantibody production.[11]

Expression and functional studies of immune cells can improve our understanding of molecular mechanisms underlying MS; recent advances in high throughput techniques, such as RNA-sequencing and microarray, have enabled simultaneous assessment of the expression of several genes. Consequently, a substantial amount of data on the expression profiles of various samples has become readily accessible to the scientific community.[12] Diagnosis of MS in its early stages is challenging because of the complex nature and heterogeneous symptoms of the disease and the absence of specific auxiliary examination markers. Therefore it is crucial to find appropriate diagnostic biomarkers for MS diagnosis.[13]

In the current study, we aimed to identify key genes and pathways in the pathogenesis of MS and evaluate their diagnostic value.

MATERIALS AND METHODS

Data Collection

We downloaded a microarray expression profiling dataset (GSE21942) from NCBI gene expression omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). The GSE21942 dataset contained expression profiles of 14 MS patients and 15 healthy controls. Expression profiling was performed using RNA extracted from peripheral blood mononuclear cells (PBMCs) of participants' blood samples. The microarray chip platform that was used in this microarray dataset was GPL570 (HG-U133_Plus_2) which includes 54675 probes.

Data Processing, Normalization, and Quality Control

Background correction and normalization were performed using the Robust Multichip Average (RMA) method[14] in R software using the affy package. Then we drew a boxplot of expression distribution of probes for all samples. Also, we performed principal component analysis (PCA), and drew a scatter plot of principal components of data to investigate the quality of data.

Differential Expression Analysis and Pathway Enrichment

Differential expression analysis was performed using the limma package in R that fits a linear model to expression data. Benjamini-Hochberg method was used for p value adjustment. Genes with log2 fold change (log2FC)>1 and adjusted p value<0.05 were considered differentially expressed genes (DEGs). Then we utilized the Enrichr online tool (https://maayanlab.cloud/Enrichr/) to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on DEGs to find crucial signaling pathways that DEGs are implicated in.

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

Recruitment of Patients and Controls

A total of 100 participants including 50 MS patients (30 males and 20 females) and 50 gender- and age-matched healthy individuals (28 males and 22 females) were recruited in this study. MS patients were diagnosed based on McDonald's criteria by a neurologist. MS patients recruited in this study were diagnosed with relapsing-remitting MS. Inclusion criteria for patients were MS diagnosis and exclusion criteria were having a positive family background, infection, and other autoimmune and metabolic diseases (e.g., diabetes). The control group was not affected by neurological or infectious and immune-related diseases. All participants signed informed consent.

Blood Sampling, RNA Extraction, and cDNA Synthesis

Five milliliters of venous blood were drawn from all study participants and collected in EDTA-containing tubes. Then the blood samples were transferred to our lab and stored at −80°C to avoid RNA degradation until their RNA was extracted. Total blood RNA was extracted using a GeneALL RNA extraction kit (GeneAll Hybrid-R Blood RNA, South Korea) according to manufacturer instructions. cDNA was synthesized from extracted RNA using an AddScript cDNA synthesis kit (AddBio, South Korea) according to the manufacturer's instructions.

Expression Study

Expression of selected genes (*BLNK*, *KRAS*, *BLK*, *CR2*, *RASGRP3*) was evaluated using real-time quantitative polymerase chain reaction (PCR). Specific primers for each gene were designed using Allele ID 7.5 (Premier Biosoft, USA). Characteristics of PCR primers used in this study are summarized in supplementary Table 1. PCR reaction solutions were prepared using Ampliqon Master Mix (Odense, Denmark) and the reactions were run in LightCycler 96 instrument (Roche, Switzerland). *GAPDH* was considered a reference gene and its expression was used to normalize the expression of other genes in each sample.

Statistical Analysis

The relative expression level of selected genes in clinical samples was calculated using the $2^{-\Delta\Delta Ct}$ method. The *t*-test was used to compare the expression level of genes in patients and controls. We corrected *p* values for multiple comparisons using the Bonferroni method in clinical samples. Then we evaluated the correlation between expression levels of selected genes in clinical samples based on the Pearson method and the correlation between gene expression and demographic characteristics of patients was appraised using the Spearman method. All the above-mentioned analyses were carried out in R software version 4.2.0. Then we performed receiver operating characteristic (ROC) analysis in GraphPad Prism software version 8 (United States), plotted an ROC curve for each gene, and computed the area under the curve (AUC) for each gene of interest to investigate the diagnostic power of expression levels of selected genes. In all analyses in this study, *p* values<0.05 were considered statistically significant.

RESULTS

Bioinformatic Studies

The GSE21942 dataset included 14 MS patients and 15 healthy controls. After background correction and normalization, the quality of data was checked and a boxplot of the expression distribution of probes for each sample (Figure 1) and a scatter plot of principal components were drawn (Figure 2). Results indicated that GSE21942 had appropriate quality so we moved forward with this dataset.

We employed the limma package to identify differentially expressed genes (log2FC>1 and adjusted *p* value<0.05) in MS patients compared with controls. We detected 266 DEGs in MS patients in comparison to controls including 183 upregulated genes and 83 downregulated genes. Then we performed KEGG pathway enrichment and the most significant signaling pathways enriched included the B cell receptor (BCR) signaling pathway, hematopoietic cell lineage, transcriptional misregulation in cancer, and Epstein-Barr virus infection. The top 10 enriched pathways are featured in Figure 3 with their corresponding *p* values.

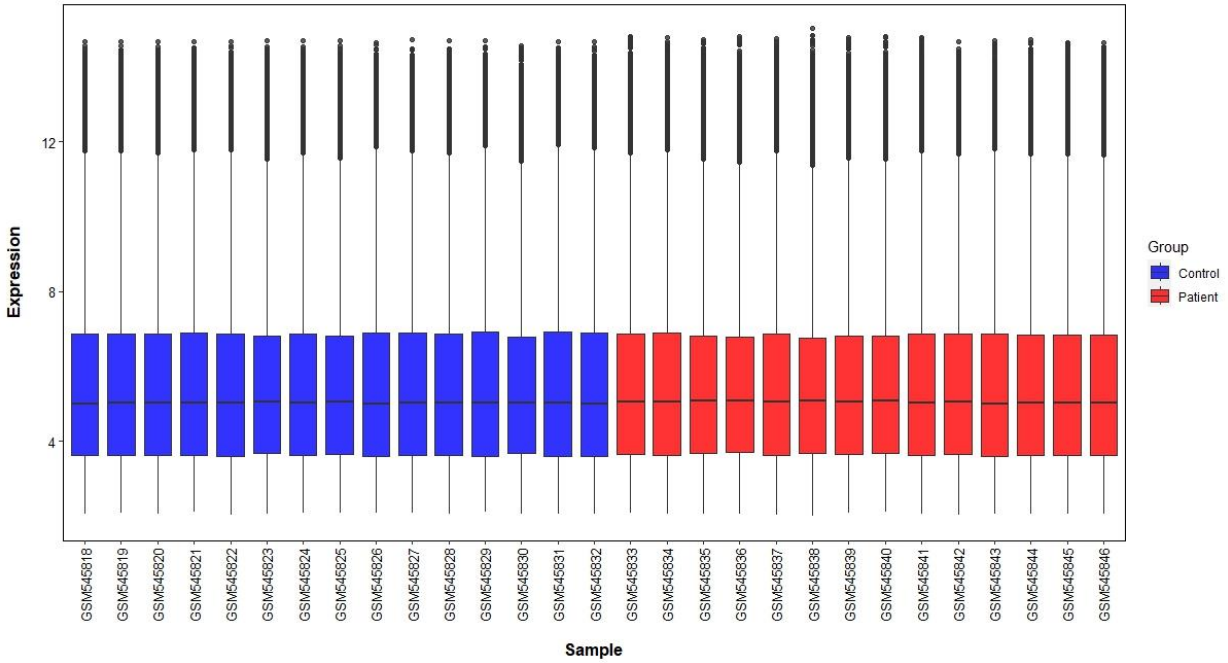

Figure 1. Box plot of gene expression distribution in every sample of microarray data after normalization. It can be understood from this box plot that gene expression distribution in different samples has approximately equivalent range and indicates that data has acceptable quality.

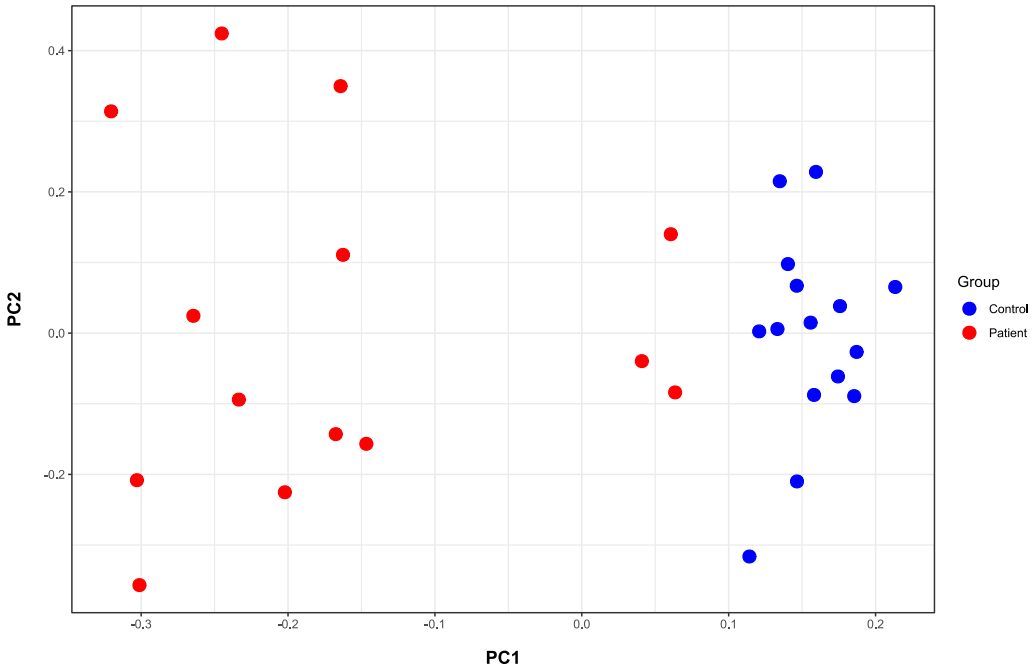

Figure 2. Scatter plot of principal components (PCs). This plot shows that the microarray dataset possesses acceptable quality; patients and controls are scattered separately based on PC1 and PC2.

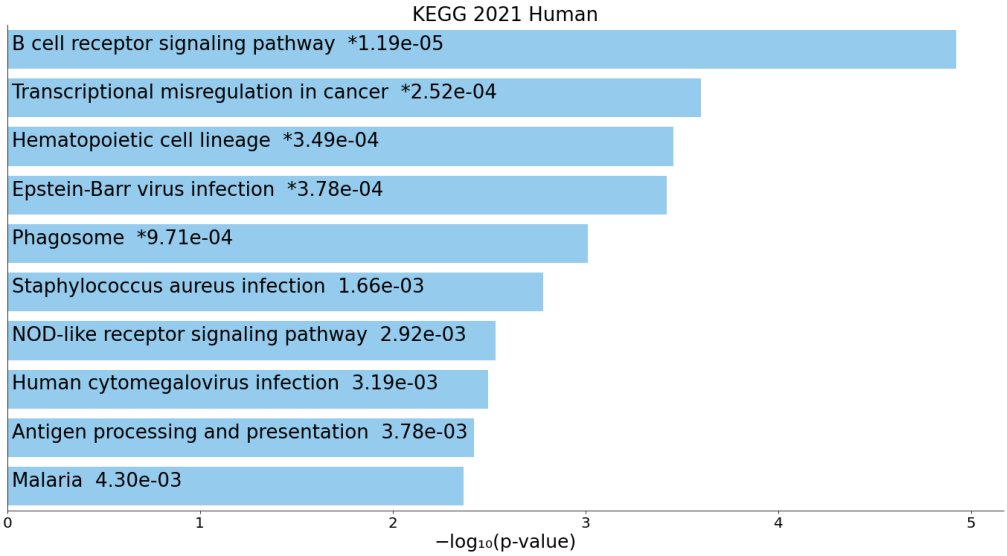

Figure 3. Top ten enriched signaling pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. As it is reflected by the figure, the B cell receptor signaling pathway has the highest odds ratio among other significantly enriched pathways.

The role of B cells in the pathogenesis of MS has not been thoroughly studied. Because of the high efficacy of B-cell depleting therapy using an anti-CD20 antibody (rituximab) in restraining MS disease activity and progression[10] and the highest odds ratio (OR) of BCR signaling pathway (OR=8.38) among other significant enriched pathways, we selected this signaling pathway for further investigation. We chose 5 genes of the BCR signaling pathway including *BLNK*, *BLK*, *CR2*, *RASGRP3*, and *KRAS* to validate in clinical samples and evaluate their diagnostic value. All of these genes except for *KRAS* were overexpressed in MS patients according to the results of microarray data analysis.

Expression Assay

Participants entered in this study included 50 MS patients and 50 gender- and age-matched normal subjects. Demographic data of study subjects are summarized in Table 1.

Expression of selected genes was evaluated in patients and controls using real-time quantitative PCR. Expression of the *CR2* gene was higher in MS patients compared with healthy controls (fold change (FC)=3.09, standard error of the mean (SEM)=0.207, $p<0.001$). Comparisons in gender subgroups also indicated that expression of *CR2* was upregulated in both male and female MS patients in comparison to their corresponding gender in the control group (Figure 4). *BLNK* was upregulated in MS patients compared to controls (FC=2.19, SEM=0.194, $p<0.001$). Gender-based analysis showed that *BLNK* expression was upregulated in male patients compared with male controls and female patients compared with female controls.

Table 1. Demographic data of study participants.

	Group		*p*
	Controls (n=50)	Patients (n=50)	
Gender			
Male	22 (44%)	20 (40%)	0.685
Gender	28 (56%)	30 (60%)	
Age (mean ± SD)	33.93 ±3.64	35.09 ±7.29	0.320

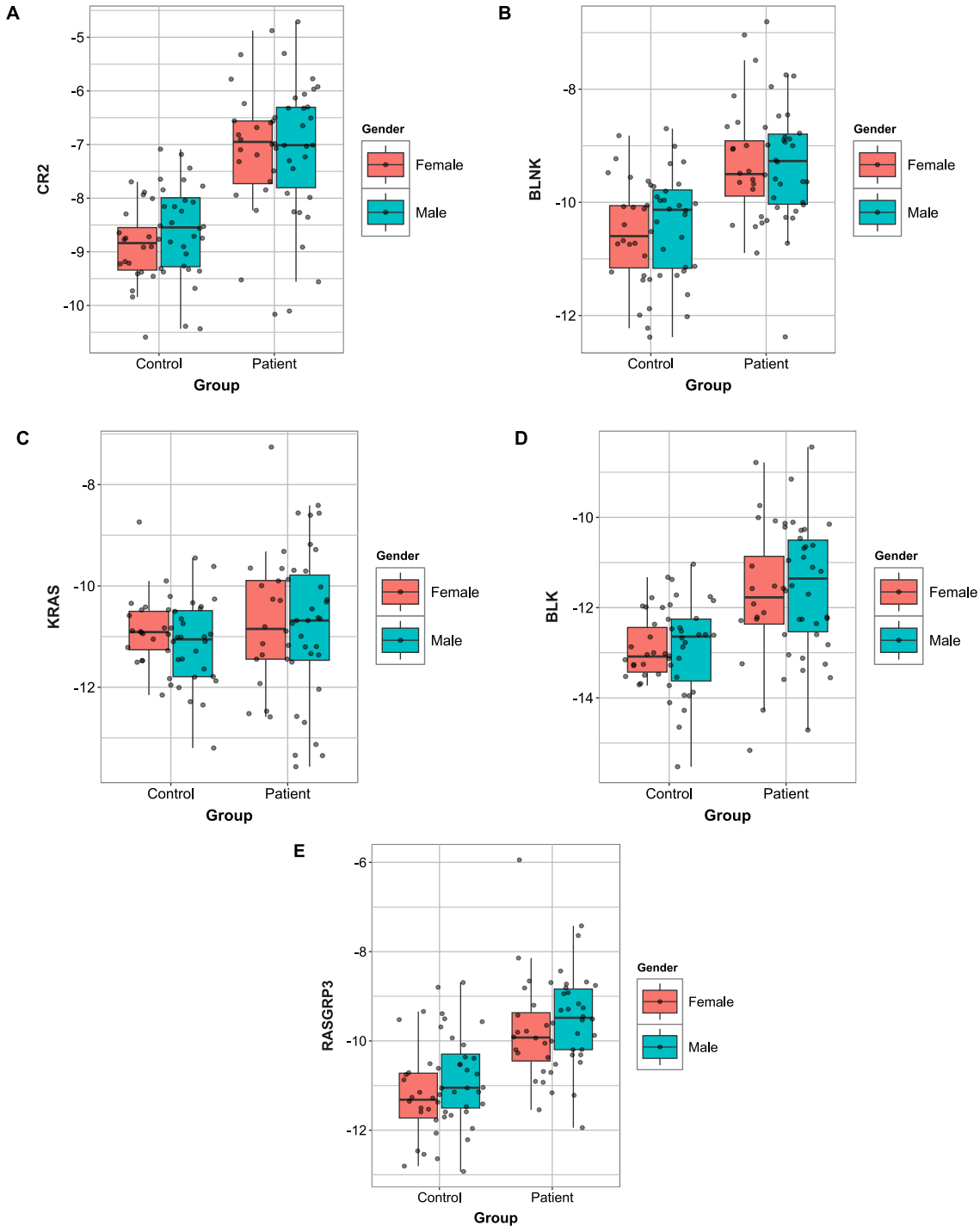

Figure 4. Relative expression of selected genes in clinical samples of multiple sclerosis (MS) patients and controls. Y axis corresponds to -ΔCt and X axis corresponds to the study group (patients and controls). Red boxes correspond to female subjects and blue boxes correspond to male individuals. A indicates the relative expression of the *CR2* gene in patients and controls. B indicates the relative expression of the *BLNK* gene in patients and controls. C indicates the relative expression of the *KRAS* gene in patients and controls. D indicates the relative expression of the *BLK* gene in patients and controls and E indicates the relative expression of the *RASGRP3* gene in patients and controls. All genes are differentially expressed in MS patients compared with controls except for the *KRAS* gene.

Similarly, the *RASGRP3* gene was overexpressed in MS patients compared with healthy controls (FC=2.59, SEM=0.209, $p<0.001$). Expression of *RASGRP3* was higher in both sexes of patients in contrast to sex-matched controls. Also, *BLK* showed a similar pattern of expression and was upregulated in MS patients compared to controls (FC=2.39, SEM=0.245, $p<0.001$). Increased expression of *BLK* was also observed in male patients compared with male controls and female patients compared with female controls. There was no statistically significant difference in the expression of *KRAS* between MS patients and controls ($p=0.283$). Even comparisons in gender subgroups showed no significant difference in *KRAS* expression levels in male and female patients in comparison to their corresponding controls. The results of expression assays of 4 genes (*CR2*, *BLNK*, *RASGRP3*, and *BLK*) in clinical samples were in line with the findings of microarray data, in other words, the results of microarray assay were validated in our clinical samples except for *KRAS* that showed no significant difference between study groups. Detailed parameters of expression analysis of each gene are shown in Table 2.

Table 2. Detailed parameters of expression analysis of selected genes in clinical samples.

Gene	Gender	Mean Difference[a] (MS vs. CTL)	Fold Change	Std. Error	Significance[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
	Total	1.626	3.09	0.207	< 0.001	1.216	2.037
CR2	Female	1.784	3.44	0.310	< 0.001	1.157	2.411
	Male	1.5	2.83	0.279	< 0.001	0.941	2.059
	Total	1.134	2.19	0.194	< 0.001	0.749	1.519
BLNK	Female	1.187	2.28	0.288	< 0.001	0.605	1.769
	Male	1.091	2.13	0.266	< 0.001	0.558	1.624
	Total	1.372	2.59	0.209	< 0.001	0.956	1.787
RASGRP3	Female	1.456	2.74	0.350	< 0.001	0.750	2.163
	Male	1.291	2.44	0.258	< 0.001	0.774	1.807
	Total	1.256	2.39	0.245	< 0.001	0.770	1.742
BLK	Female	1.103	2.15	0.369	0.005	0.358	1.849
	Male	1.358	2.56	0.331	< 0.001	0.694	2.022
	Total	0.247	–	0.228	0.283	–0.206	0.700
KRAS	Female	0.173	–	0.317	0.588	–0.467	0.813
	Male	0.314	–	0.323	0.336	–0.334	0.961

MS: Multiple Sclerosis, CTL: Control, Std. Error: standard error of mean

a. Mean difference corresponds to the $-\Delta\Delta Ct$ value. b. Adjustment for multiple comparisons: Bonferroni

Correlation and ROC Analysis

The pairwise correlation between expression levels of different genes in clinical samples was evaluated using the Pearson method. Significant correlations were observed between different genes in both patients and controls. The strongest correlation was between *BLNK-BLK* (R=0.82, $p<0.001$) and *CR2-BLK* (R=0.77, $p<0.001$) in patients (Figure 5) and between *BLNK-BLK* (R=0.70, $p<0.001$) and *CR2-BLNK* (R=0.68, $p<0.001$) in controls (Figure 6). Also, we appraised the correlation

of demographic characteristics of patients with expression levels of genes in patient groups using the Spearman method. We identified no significant correlation between the demographic characteristics of patients and the expression level of any gene.

To evaluate the diagnostic power of each gene, we illustrated the ROC curve and computed the AUC for each gene (Figure 7). *CR2* gene was most efficient in differentiating MS patients from the control group with AUC=0.87, specificity=0.80, and sensitivity=0.82. The second robust gene in the differentiation of cases from controls was *RASGRP3* with AUC=0.83, specificity=0.76, and sensitivity=0.82. Also, the *BLNK* gene had acceptable efficacy in separating MS cases from controls with AUC=0.80, specificity=0.74, and sensitivity=0.78. Finally, the *BLK* gene could distinguish MS cases from controls with AUC=0.77, specificity=0.78, and sensitivity=0.80. *KRAS* could not significantly differentiate patients and controls.

We found that combinations of genes could drastically augment diagnostic power (Figure 8). The combination of 3 genes with the highest values of AUC (*CR2*, *RASGRP3*, and *BLNK*) resulted in a noticeable increase in diagnostic power with AUC=0.94. In addition, the combination of 4 genes including *CR2*, *RASGRP3*, *BLNK*, and *BLK* even yielded higher differentiation ability (AUC=0.96). Detailed information on ROC analyses is shown in Table 3. Taken together, these results suggest that the selected genes have acceptable diagnostic power and may be implicated in the pathogenesis of MS.

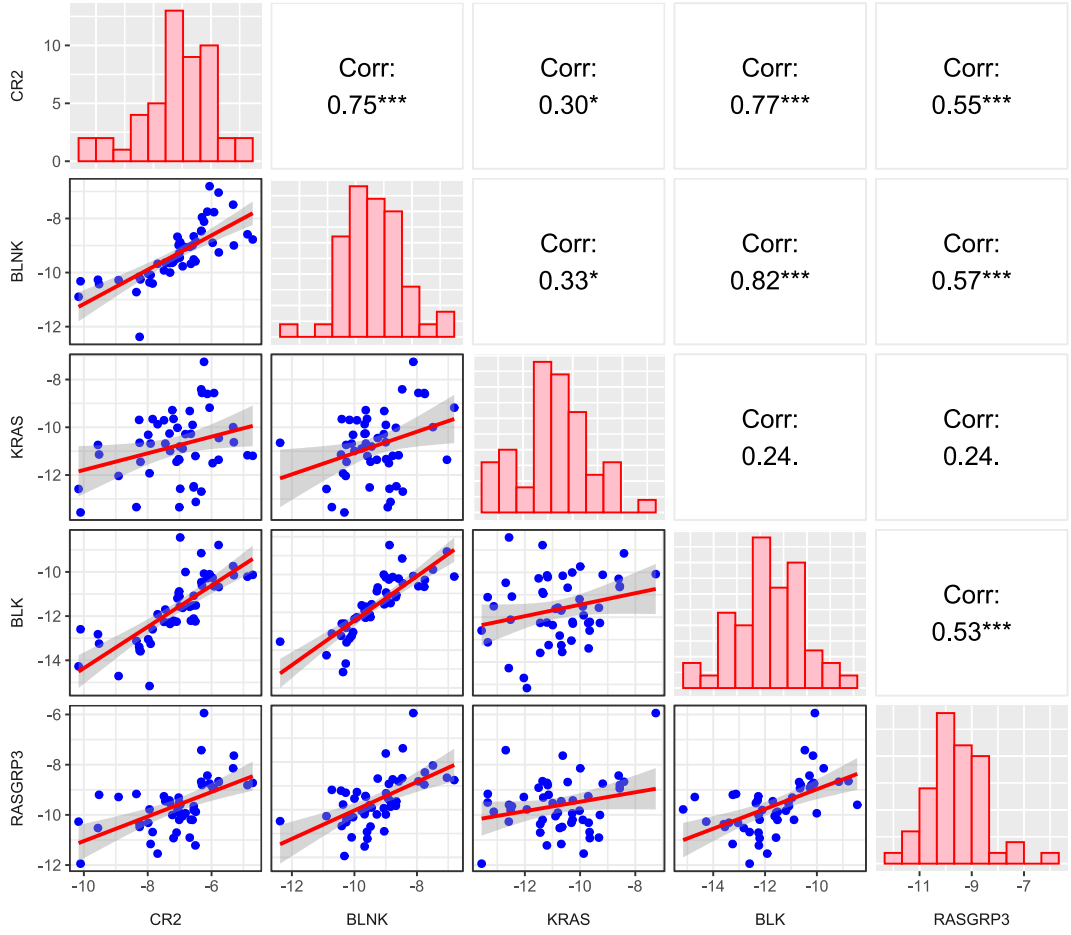

Figure 5. Pairwise correlation between expression levels of selected genes in clinical samples of multiple sclerosis patients. *p<0.05, **p<0.01, **p<0.001. Corr; correlation coefficient

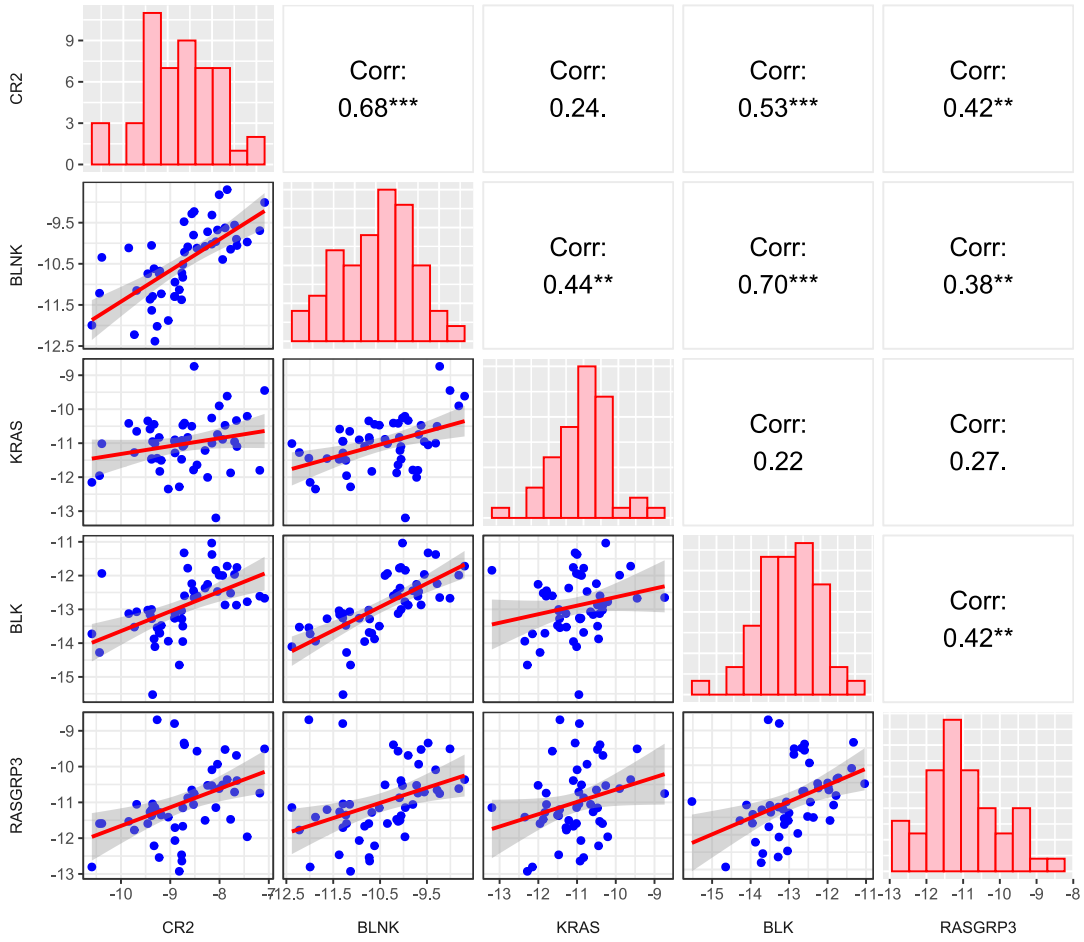

Figure 6. Pairwise correlation between expression levels of selected genes in clinical samples of control individuals. *p<0.05, **p<0.01, **p<0.001. Corr; correlation coefficient

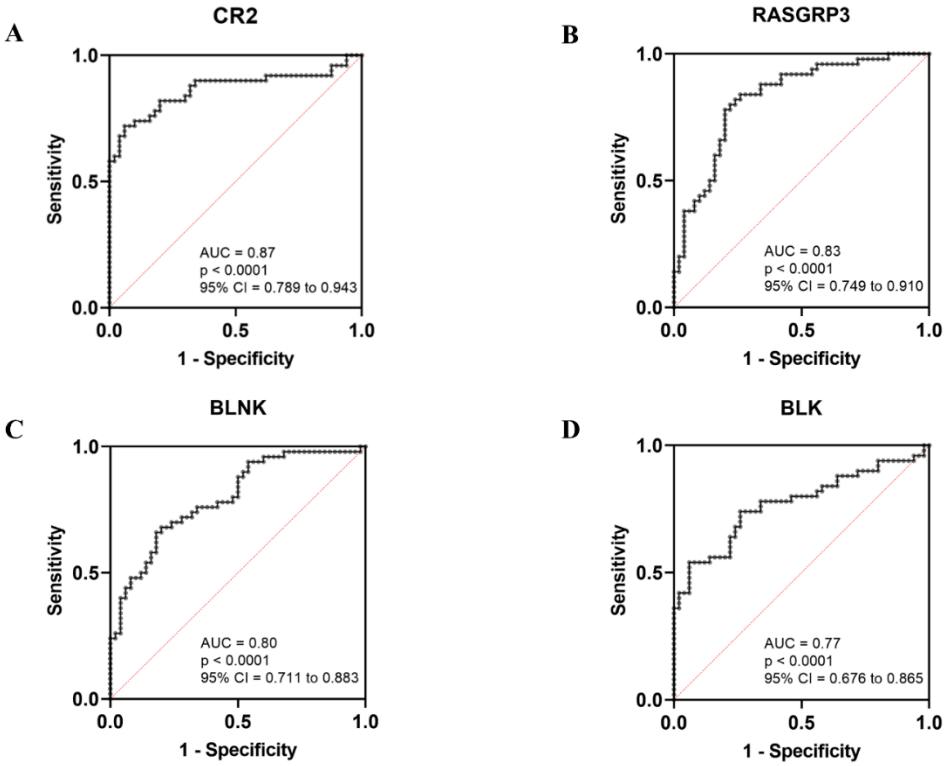

Figure 7. Receiver operating characteristic (ROC) curves of selected genes with significant differential expression in multiple sclerosis patients. A is the ROC curve of the *CR2* gene; B is the ROC curve of the *RASGRP3* gene; C is the ROC curve of the *BLNK* gene; D is the ROC curve of the *BLK* gene. AUC; Area Under Curve, CI; Confidence Interval

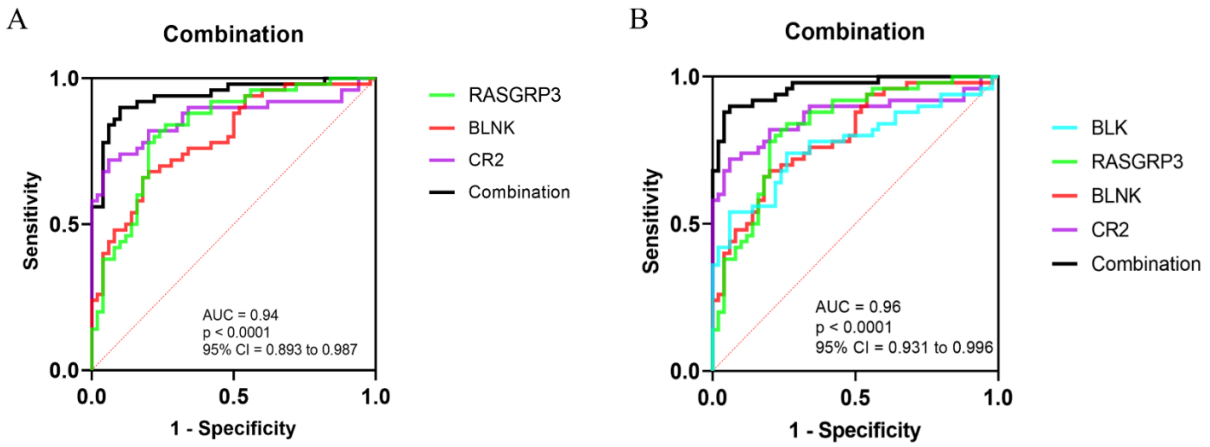

Figure 8. Combination Receiver operating characteristic curves. A: combination of three genes (*CR2*, *RASGRP3*, *BLNK*) with an area under curve (AUC) of at least 0.80. B: the combination of all 4 genes with differential expression in multiple sclerosis cases (*CR2*, *RASGRP3*, *BLNK*, and *BLK*). CI; Confidence Interval

Table 3. Detailed information of receiver operating characteristic **analyses**

Gene	AUC*	Sensitivity	Specificity	95% CI**	*p*
CR2	0.87	0.82	0.80	0.789 to 0.943	< 0.0001
BLNK	0.8	0.78	0.74	0.711 to 0.883	< 0.0001
RASGRP3	0.83	0.82	0.76	0.749 to 0.910	< 0.0001
BLK	0.77	0.80	0.78	0.676 to 0.865	< 0.0001
CR2* + *BLNK* + *RASGRP3	0.94	0.90	0.90	0.893 to 0.987	< 0.0001
CR2* + *BLNK* + *RASGRP3*+ *BLK	0.96	0.92	0.86	0.931 to 0.996	< 0.0001

*Area Under Curve, **Confidence Interval

DISCUSSION

In the current study we identified 266 differentially expressed genes in MS patients compared with healthy controls through analyzing a microarray gene expression profiling dataset (GSE21942). We performed KEGG pathway enrichment on DEGs and selected the BCR signaling pathway and 5 genes (*CR2*, *BLNK*, *BLK*, *RASGRP3*, and *KRAS*) involved in this signaling pathway for further investigation and validation in clinical samples.

Despite the evidence that indicates B cells can be involved in MS pathogenesis their role in MS has been overlooked and is not fully understood. It has been established that B cells exist in brain lesions of MS patients. Additionally, the detection of oligoclonal bands in the cerebrospinal fluid of around 90% of MS patients underscores the role of B cells in this autoimmune disease.[15] It has been shown that B-cell depleting therapy using anti-CD20 antibodies is highly efficient in restraining disease activity and progression. Targeting B cells also prevents activation of T cells and results in reduced proinflammatory cytokines.[10] Even deep investigation of other approved treatments for MS such as alemtuzumab, cladribine, fingolimod, interferon-β, and dimethyl fumarate have demonstrated that these medications target B cells (especially memory B cells) as well as T cells and alleviate MS progression.[16] Also, Magliozzi and colleagues have demonstrated that there are tertiary lymphoid structures in the brain of MS patients that are located in the vicinity of brain lesions. These tertiary structures are rich in T cells and B cells and contribute to inflammatory processes causing neurodegeneration in cortical areas.[17] In addition, it has been demonstrated that B cells and plasma cells exist in CNS lesions of MS patients.[18] B cells can be involved in MS pathogenesis through different pathways including antigen presentation to autoreactive T cells, secretion of inflammatory cytokines and chemokines, and autoantibody production.[11] All of these facts emphasize the potential role of B cells in the pathogenesis of MS. Based on these findings we were convinced to select the BCR signaling pathway and genes involved in this pathway for further investigations.

We investigated the expression of *CR2*, *BLNK*, *BLK*, *RASGRP3*, and *KRAS* in the peripheral blood of 50 MS patients compared with 50 age and gender-matched controls. Expression of all genes except KRAS were significantly upregulated in MS cases in comparison to controls. Comparisons in gender subgroups showed similar patterns and we observed that expression of *CR2*, *BLNK*, *BLK*, and *RASGRP3* was higher in both genders of MS patients compared with their gender-matched controls. Also, the ROC curve illustration indicated that all four genes with differential expression in MS cases had acceptable diagnostic power and *CR2* had the highest AUC value in differentiating MS patients from normal subjects.

CR2 is a membrane protein that is expressed on B cells' surface and acts as a receptor for EBV (a well-known risk factor of MS). It has been demonstrated that a common haplotype in the *CR2* gene including 3 single nucleotide variants (SNVs) is associated with the risk of lupus, an autoimmune disease.[19] *CR2* plays a key role in B cell activation and potentiates signal transduction by BCR through binding with C3d bound to antigen.[20] We found that *CR2* had increased expression in MS patients, in accordance with our results Lindblom et al. reported

high amounts of *CR2* in the cerebrospinal fluid of MS patients compared with controls. Furthermore, they suggested that *CR2* was implicated in the regulation of complement activation through modulating the C3 component of the complement system.[21] In another study, researchers indicated that *CR2* could contribute to the internalization and presentation of self-antigen myelin basic protein (MBP), an MS-related autoantigen, by B cells. They discovered that *CR2* contributes to this process in a complement-dependent manner through the deposition of C3 and C1q along with MBP on B cells.[22] So, *CR2* can be involved in MS pathogenesis by assisting B cells in presenting self-antigens. Also, Hu et al. in a study evaluated the effect of inhibition of *CR2* on experimental autoimmune encephalomyelitis (EAE, a mouse model of autoimmune neurodegenerative disease). They observed that inhibition of *CR2* can alleviate both acute and chronic forms of EAE by blocking the activation of complement.[23]

In a microarray gene expression profiling study, altered expression of *BLK* and *BLNK* were reported in MS patients compared with controls. Furthermore, a significant correlation was observed between the cognitive function of MS patients and expression levels of *BLK* and *BLNK*.[24] Similarly, we identified high expression of these 2 genes in MS cases in our clinical samples and the microarray dataset we analyzed (GSE21942). *BLK* also known as B-lymphoid tyrosine kinase is a non-receptor protein tyrosine kinase and belongs to the Src family of tyrosine kinases. *BLK* is mainly expressed in B cell lineage but its expression has also been found at lower amounts in non–B-cell lineage such as pancreatic β-cells and thymocytes. *BLK* is implicated in B cell development and BCR signaling.[25] BCR stimulation leads to the activation of *BLK* which subsequently phosphorylates downstream molecules and contributes to the activation of downstream pathways such as the PI3K pathway.[26] Also, *BLK* interacts with *BANK1* and facilitates *BANK1* and phospholipase C gamma 2 (PLCg2) binding that connects BCR-mediated signaling to downstream mediators.[27] It has been shown that several variants in the *BLK* gene are associated with different autoimmune diseases. For instance, Hom et al. demonstrated that rs13277113 polymorphism in the *BLK* gene is associated with susceptibility to systemic lupus erythematosus (SLE).[28] Also, *BLK* variants are correlated with the risk of rheumatoid arthritis (RA)[29] and other autoimmune disorders such as systemic sclerosis[30] and Sjögren's syndrome.[31] *BLNK* also known as B cell linker is an adapter protein in BCR signaling. After stimulation of BCR, *BLNK* gets phosphorylated and recruits Syk and Btk kinases and mediates activation of downstream signaling pathways such as the PLC-γ2 pathway.[32] Liubchenko and colleagues showed that *BLNK* phosphorylation in B cells obtained from RA patients was higher than that in B cells obtained from healthy controls.[33] In a recent study, researchers indicated that *BLNK*, *BLK*, and other components of the BCR signaling pathway are activated in a mouse model of lupus and result in abnormal development of B cells in this disease.[34] Also, it has been shown that individuals carrying *BLK* risk alleles possess an increased number of isotype-switched memory B cells. In addition, B cells are overactive and T cells have more stimulatory capacity.[35] Therefore, aberrant activation of *BLNK* and *BLK* may be implicated in MS pathogenesis.

Ras guanyl nucleotide-releasing proteins (RasGRPs) are a protein family serving as guanyl nucleotide exchange factors and play a key role in the activation of Ras GTPases. RASGRP family members including *RASGRP1*, *RASGRP2*, *RASGRP3*, and *RASGRP4* are mainly expressed in blood cells and their dysregulation has been observed in autoimmune diseases and blood neoplasms.[36] It has been shown that *RASGRP1* bridges T cell receptor signaling to downstream Ras signaling and in B cells *RASGRP1* and *RASGRP3* are involved in BCR-induced activation of downstream Ras, with the special role of *RASGRP3* in maintaining basal levels of Ras-GTP in resting B cells.[37] B cells carrying null mutations in *RASGRP3* (*RASGRP3*-/- B cells) have attenuated Ras-Erk signaling in both basal and BCR-induced states; these findings underline the important role of *RASGRP3* in BCR signaling.[38] In a recent study, researchers indicated that *RASGRP3* was upregulated in PBMCs and purified B cells of SLE patients compared with controls and *RASGRP3* expression level had a positive correlation with SLE disease activity index (SLEDAI). Furthermore, they discovered that *RASGRP3* knockdown prevented activation of Erk1/2 signaling and restricted the production of pro-inflammatory cytokines.[39] Similarly, we found *RASGRP3* overexpression in MS patients compared with controls. So, upregulation of *RASGRP3* might be involved in MS pathogenesis through mediating overactivation of BCR signaling. In a Genome-wide association study (GWAS), the association of rs13385731 SNV in *RASGRP3* with SLE susceptibility

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

was demonstrated[40] and in another study by Wang et al. this result was replicated and authors showed that rs13385731 SNV in *RASGRP3* was associated with SLE risk in Caucasian populations.[41] In a study, Jelcic et al, represented an astonishing finding about the discovery of a novel MS-related autoantigen. They discovered that *RASGRP2*, another member of the RASGRP family, that has high expression in B cells of MS patients and cortical neurons, can be presented by these cells to autoreactive T helper cells and lead to CNS inflammation upon migration of stimulated T cells to brain.[42] Together these findings underline the critical role of the *RASGRP* family in BCR signaling and autoimmunity.

Limitations of the current study would be a relatively small sample size and a lack of functional investigations of selected pathways and genes. The results of this study and the diagnostic power of selected genes need to be validated in a larger sample size. Also, functional studies are needed to investigate the role of each gene in B cell receptor signaling activity and disease behavior. We will address these limitations in our future studies.

To sum up, in this study we found the B cell receptor signaling pathway as a key pathway in MS and indicated that expression levels of *CR2*, *BLK*, *BLNK*, and *RASGRP3* from this pathway were higher in peripheral blood of MS patients than that in healthy controls. Also, we discovered that above mentioned genes had high diagnostic power in differentiating MS patients from healthy controls. Based on these findings BCR signaling pathway might be considered as a crucial pathway in MS pathogenesis and also each of these genes would be considered as a potential diagnostic biomarkers and therapeutic targets in MS. Further functional and replication studies are needed to validate these qualities.

STATEMENT OF ETHICS

The current research was approved by Research Ethics Committees of Directorate of Health, Rescue and Treatment of Police Headquarter of Islamic Republic of Iran (approval ID:IR.SBMU.TEB. POLICE.REC.1402.003).

FUNDING

This research was funded by the Research Center for Life & Health Sciences & Biotechnology of the Police.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

We acknowledge Research Center for Life & Health Sciences & Biotechnology of the Police for their financial support.

REFERENCES

1. Dobson R, Giovannoni G. Multiple sclerosis–a review. Eur J Neurol. 2019;26(1):27-40.
2. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS. Mult Scler. 2020;26(14):1816-21.
3. Almasi-Hashiani A, Sahraian MA, Eskandarieh S. Evidence of an increased prevalence of multiple sclerosis: a population-based study of Tehran registry during 1999–2018. BMC Neurol. 2020;20:1-7.
4. Lassmann H. Multiple sclerosis pathology. Cold Spring Harb Perspect Med. 2018;8(3).
5. Van der Mei I, Lucas RM, Taylor B, Valery P, Dwyer T, Kilpatrick TJ, et al. Population attributable fractions and joint effects of key risk factors for multiple sclerosis. Mult Scler. 2016;22(4):461-9.
6. Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmun Rev. 2010;9(5):A387-A94.
7. Mosca L, Mantero V, Penco S, La Mantia L, De Benedetti S, Marazzi MR, et al. HLA-DRB1* 15 association with multiple sclerosis is confirmed in a multigenerational Italian family. Funct Neurol. 2017;32(2):83.
8. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. Lancet Neurol. 2010;9(7):727-39.
9. Goodin DS. The pathogenesis of multiple sclerosis. Clin Exp Neuroimmunol. 2015;6:2-22.
10. Greenfield AL, Hauser SL. B-cell Therapy for Multiple Sclerosis: Entering an era. Ann Neurol. 2018;83(1):13-26.
11. Jelcic I, Sospedra M, Martin R. When a T cell engages a B cell: novel insights in multiple sclerosis. Swiss Med Wkly. 2020;150(3536):w20330-w.
12. Kemppinen A, Kaprio J, Palotie A, Saarela J. Systematic review of genome-wide expression studies in multiple sclerosis. BMJ Open. 2011;1(1):e000053.

13. Xu ZB, Feng X, Zhu WN, Qiu ML. Identification of key genes and microRNAs for multiple sclerosis using bioinformatics analysis. Medicine. Medicine (Baltimore). 2021;100(48).
14. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4(2):249-64.
15. Sospedra M. B cells in multiple sclerosis. Curr Opin Neurol. 2018;31(3):256-62.
16. Li R, Patterson KR, Bar-Or A. Reassessing B cell contributions in multiple sclerosis. Nat Immunol. 2018;19(7):696-707.
17. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain. 2007;130(4):1089-104.
18. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain. 2009;132(5):1175-89.
19. Wu H, Boackle SA, Hanvivadhanakul P, Ulgiati D, Grossman JM, Lee Y, et al. Association of a common complement receptor 2 haplotype with increased risk of systemic lupus erythematosus. PNAS. 2007;104(10):3961-6.
20. Rickert RC. Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex. Curr Opin Immunol. 2005;17(3):237-43.
21. Lindblom RP, Aeinehband S, Ström M, Al Nimer F, Sandholm K, Khademi M, et al. Complement Receptor 2 is increased in cerebrospinal fluid of multiple sclerosis patients and regulates C3 function. Clin Immunol. 2016;166:89-95.
22. Brimnes MK, Hansen BE, Nielsen LK, Dziegiel MH, Nielsen CH. Uptake and presentation of myelin basic protein by normal human B cells. PLoS One. 2014;9(11):e113388.
23. Hu X, Tomlinson S, Barnum SR. Targeted inhibition of complement using complement receptor 2-conjugated inhibitors attenuates EAE. Neurosci Lett. 2012;531(1):35-9.
24. Turkoglu R, Yilmaz V, Ozdemir O, Akbayir E, Benbir G, Arsoy E, et al. Peripheral blood B cell subset ratios and expression levels of B cell-associated genes are altered in benign multiple sclerosis. Mult Scler Relat Disord. 2021;52:103019.
25. Simpfendorfer KR, Olsson LM, Manjarrez Orduño N, Khalili H, Simeone AM, Katz MS, et al. The autoimmunity-associated BLK haplotype exhibits cis-regulatory effects on mRNA and protein expression that are prominently observed in B cells early in development. Hum Mol Genet. 2012;21(17):3918-25.
26. Saouaf SJ, Mahajan S, Rowley RB, Kut SA, Fargnoli J, Burkhardt AL, et al. Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. PNAS. 1994;91(20):9524-8.
27. Bernal-Quirós M, Wu YY, Alarcón-Riquelme ME, Castillejo-López C. BANK1 and BLK act through phospholipase C gamma 2 in B-cell signaling. PLoS One. 2013;8(3):e59842.
28. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S, et al. Association of systemic lupus erythematosus with C8orf13–BLK and ITGAM–ITGAX. NEJM. 2008;358(9):900-9.
29. Deshmukh HA, Maiti AK, Kim-Howard XR, Rojas-Villarraga A, Guthridge JM, Anaya JM, et al. Evaluation of 19 autoimmune disease-associated loci with rheumatoid arthritis in a Colombian population: evidence for replication and gene-gene interaction. J Rheumatol. 2011;38(9):1866-70.
30. Gourh P, Agarwal SK, Martin E, Divecha D, Rueda B, Bunting H, et al. Association of the C8orf13-BLK region with systemic sclerosis in North-American and European populations. J Autoimmun. 2010;34(2):155-62.
31. Nordmark G, Kristjansdottir G, Theander E, Appel S, Eriksson P, Vasaitis L, et al. Association of EBF1, FAM167A (C8orf13)-BLK and TNFSF4 gene variants with primary Sjögren's syndrome. Genes Immun. 2011;12(2):100-9.
32. Wen Y, Jing Y, Yang L, Kang D, Jiang P, Li N, et al. The regulators of BCR signaling during B cell activation. Blood Sci. 2019;1(02):119-29.
33. Liubchenko GA, Appleberry HC, Striebich CC, Franklin KE, Derber LA, Holers VM, et al. Rheumatoid arthritis is associated with signaling alterations in naturally occurring autoreactive B-lymphocytes. J Autoimmun. 2013;40:111-21.
34. Tang W-Y, Zhang Y-H, Zhang Y-S, Liao Y, Luo J-S, Liu J-H, et al. Abnormal thymic B cell activation and impaired T cell differentiation in pristane-induced lupus mice. Immunol Lett. 2021;231:49-60.
35. Simpfendorfer KR, Armstead BE, Shih A, Li W, Curran M, Manjarrez-Orduño N, et al. Autoimmune disease–associated haplotypes of BLK exhibit lowered thresholds

for B cell activation and expansion of Ig class-switched B cells. Arthritis Rheumatol. 2015;67(11):2866-76.
36. Stone JC. Regulation and function of the RasGRP family of Ras activators in blood cells. Genes Cancer. 2011;2(3):320-34.
37. Coughlin JJ, Stang SL, Dower NA, Stone JC. The role of RasGRPs in regulation of lymphocyte proliferation. Immunol Lett. 2006;105(1):77-82.
38. Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. J Immunol. 2005;175(11):7179-84.
39. An XJ, Xia Y, Li J, Dong LY, Wang YJ, Yang J, et al. RasGRP3 in peripheral blood mononuclear cells is associated with disease activity and implicated in the development of systemic lupus erythematosus. Am J Transl Res. 2019;11(3):1800.
40. Han JW, Zheng HF, Cui Y, Sun LD, Ye DQ, Hu Z, et al. Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat Genet. 2009;41(11):1234-7.
41. Wang C, Ahlford A, Järvinen TM, Nordmark G, Eloranta ML, Gunnarsson I, et al. Genes identified in Asian SLE GWASs are also associated with SLE in Caucasian populations. Eur J Hum Genet. 2013;21(9):994-9.
42. Jelcic I, Al Nimer F, Wang J, Lentsch V, Planas R, Jelcic I, et al. Memory B cells activate brain-homing, autoreactive CD4+ T cells in multiple sclerosis. Cell. 2018;175(1):85-100. e23.