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Research Paper: Gene Expression of *CD226* and Its Serum Levels in Patients With Multiple Sclerosis

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ABSTRACT

Background: Recent studies have found some genetic variants as a risk factor for autoimmune diseases such as Multiple Sclerosis (MS). Cluster of Differentiation 226 (*CD226*) is one of the risk factors for MS.

Objectives: The present study aimed to evaluate the gene expression of *CD226*, and its protein serum level in peripheral blood samples of MS patients and healthy individuals.

Materials & Methods: A total of 30 individuals with MS and 30 healthy individuals, as controls, referred to Kashani Hospital of Isfahan, Iran. *CD226* expression at the transcript level and serum protein levels were measured by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays, respectively. Statistical analyses were performed by Shapiro-Wilk test and nonparametric tests in SPSS.

Results: The present study showed no significant differences in the gene expression of *CD226* (P=0.341). The mean serum protein level of *CD226* was not different between the patients and the controls (P=0.978).

Conclusion: Overall, *CD226* expression has no diagnostic usefulness in MS at either the transcript or serum level.

Keywords: Multiple Sclerosis, Demyelinating diseases, Gene expression, Cluster of Differentiation 226 (*CD226*) antigen

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Highlights

- There is no significant difference in the level of *CD226* gene expression in peripheral blood sample of new cases of relapsing remitting MS patients and healthy people.

- Assessing gene expression showed no significant changes in *CD226* protein expression levels.

Plain Language Summary

Multiple Sclerosis (MS) is one of the most common causes of primary neurological disability in young people, and its prevalence rises with increasing age. Studies have found some genetic variants as a risk factor for MS. The present study aimed to evaluate the gene expression of *CD226*, and its protein serum level in peripheral blood samples of MS patients and healthy individuals. In the present study, we found no significant expression changes of *CD226* in the blood samples of patients with MS. Thus, measuring the expression of *CD226* gene is questionable as a biomarker for the diagnosis or improvement of MS.

Introduction

Multiple Sclerosis (MS) is a chronic disabling inflammatory, and demyelinating disease, caused by immune responses directed against myelin proteins and progressive axonal loss in the Central Nervous System (CNS) [1]. MS is one of the most common causes of primary neurological disability in young people, and its incidence increases in late adolescence [2]. It is most prevalent in the late 20's and early 30's [3].

Females are affected approximately 2.5 times more than males [4]. The environmental, genetic, and epigenetic factors explain the risk for developing MS [5]. There are different relationships between the prevalence of MS and environmental factors, including sun exposure, vitamin D deficiency, and Epstein-Barr Virus (EBV) [6]. Importantly, these environmental factors can affect pathogenetic pathways and some of them can have a modifying impact [7].

MS is not classified as a genetic disease. However, there is genetic risk that may be inherited in MS [8]. Recent Genome-Wide Association Study (GWAS) has identified a total of 110 distinct genetic regions associated with MS [9]. *HLA-DRB1*1501* allele is the first known potential genetic risk factor, with 14%–30% prevalence in countries with high MS risk [10]. CD4+T helper cells regulate appropriate cellular and humoral immune responses to a broad range of pathogens and get involved in many diseases progress. The balance of the earliest determined CD4+T helper cell subsets, Th1 and Th2, play an important role in autoimmune diseases [11]. Dysregulated balance of Th1 and Th2 cells can cause MS. Th1 cells are considered initially as the principal pathogenic T cells in MS [12].

The prevalence of MS extensively varies in different geographic regions, races, and genders [13]. There has been a significant concern for the epidemiology of MS in Iran during the last decade. The ratio of MS has been increasing rapidly in Iran, particularly among females and in Isfahan Province [14]. MS is a variable and unpredictable disease that places a substantial burden on patients, their families, and the health service [15].

Considerable progress has been made in prevention, improvement, and reduction of the severity of MS attacks. The expression of Cluster of Differentiation 226 (*CD226*) can be one of the contributing factors, especially in the Iranian population with MS. Genome-wide association study in autoimmune patients has identified allelic variants in some T cell costimulatory molecular pathways as genetic risk factors in disease pathogenesis. This includes allelic variants in *CD226* (DNAX Accessory Molecule1 [DNAM-1], DNAX Accessory Molecule-1), located at 18q22.3 gene encodes a 67 kDa [336 amino acid (aa)] cell surface membrane protein with 2 immunoglobulin V set domains (aa31–aa125 and aa135–aa240) with an extracellular region [16, 17].

CD226 is expressed on the majority of Natural Killer (NK) cells, T cells, monocytes, and platelets [18]. This molecule has a role in enhancing the cytotoxic function of NK cells [19], demonstrating that *CD226* is associated with Leukocyte Function Antigen 1 (LFA-1) to induce IFN-γ production in naive CD4+T cells [16]. *CD226* is one of the immunoglobulin super family members and

binds 2 different cell surface ligands, including poliovirus receptor (CD155) and Nectin-2 (CD112) [20].

CD112 and Necl-5 (CD155) are CAM (Costimulatory Activating Molecule) members, that form homodimers (for nectin-2) or heterodimers in their functions for cell adhesion [18]. The interaction of DNAM-1 (*CD226*) with its ligands is implicated in the functions of a variety of immune cells. Nectin-2 can stimulate the reaction of NK cells and cytolytic T lymphocytes through its interaction with DNAM-1 [21]. *CD226* costimulatory signals potentially promote Th1 differentiation [22], but it cannot lead to differentiation of neither Th2 nor Th0 cells, enhancing IFN-γ production by naive T cells [23].

Another study reported that knockdown of *CD226* on human T cells resulted in a decrease in T-bet and IFN-γ expression. However, the role of *CD226* on Th2 and Th17 cells remains unknown [23]. The expression of DNAM-1 in Experimental Autoimmune Encephalomyelitis (EAE) and DNAM-1 were exhibited in the skin of patients with Systemic Sclerosis (SSc) [24]. *CD226* is involved in the up-regulation of T cells. Treatment with anti-*CD226* in vivo results in a significant reduction of Th1 cell expansion and in the induction of antigen presenting cells that prohibit T cell activation [25].

In total, *CD226* could be an important biomarker in differentially regulating the pro-inflammatory (Th1/Th17)/anti-inflammatory (Th2) balance, indicating that the *CD226* could be targeted in therapeutic approaches to autoimmune diseases like MS [17]. Therefore, the present study aimed to evaluate gene expression level of *CD226* and its serum levels in patients with MS.

Materials and Methods

Study participants

A total of 30 healthy individuals and 30 new cases of Relapsing-Remitting MS (RRMS) patients diagnosed according to McDonald's criteria were included in this study. The exclusion criteria for selecting healthy controls were as follows: Suffering from any autoimmune disease, previous organ transplantation and suffering from any inflammatory disease according to the results of erythrocyte sedimentation rate and C-reactive protein. None of the patients experienced relapse within 3 months prior to the onset of the study and were not on corticosteroid agents for at least 3 months before the onset of the research. Patients on corticosteroid therapy, and immunosuppressive drugs were excluded due to the possible effect of these agents on *CD226* expression and its serum level. Table 1 presents the demographic characteristics of the participants.

Determination of *CD226* mRNA expression levels

Blood samples (3 mL) were collected from all participants using an EDTA collection tube. Total RNA was extracted from the whole blood samples using a Total RNA Extraction Mini Kit (Yekta Tajhiz, Tehran, Iran). After isolation, the quality of RNA was checked by gel electrophoresis, and RNA quantity was measured using nanodrop (OD 260 nm) (Nanophotometer Pipette, Helmholtz, Nauenberg, Germany).

At the reverse transcription step, 5 ng of total RNA was used to synthesize the complementary DNA with oligo (dT) primer using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative real-time PCR analyses were performed using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) and Step One Plus Real-Time PCR System (Applied Biosystems).

β-Actin Gene (*ACTB*) was used as an endogenous control and samples were run in triplicate. Specific primers for *CD226* was obtained from the report of Ye Xal. et al. and primers for *ACTB* as the housekeeping gene was designed by Allele ID 7.6 and BLAST (NCBI online server) [26]. Table 2 lists the sequences of primers. The relative amount of target mRNA expression was estimated by the comparative $2^{-\Delta\Delta CT}$ method which normalizes the copy number of the target mRNA to that of an endogenous reference gene (*ACTB*) [27].

Table 1. Demographic characteristics in the study groups

Characteristic	Group	
	Case	Control
Female/Male	24/6	24/6
Age, y	28.97±1.24	31±1.37

Age is represented as Mean±SD.

Table 2. Sequences of primers

Primer Name	Primer Sequence	Amplicon Size (bp)
CD226 F	5´CGTGATGAGATTGACTGTAGCCGA3´	220 (39)
CD226 R	5´GGGTGCCTTCTGTGTATCCCAG3´	
ACTB F	5´ CCACCCATGGCAAATTCCATGGCA3´	180
ACTB R	5´TCTAGACGGCAGGTCAGGTCCACC 3´	

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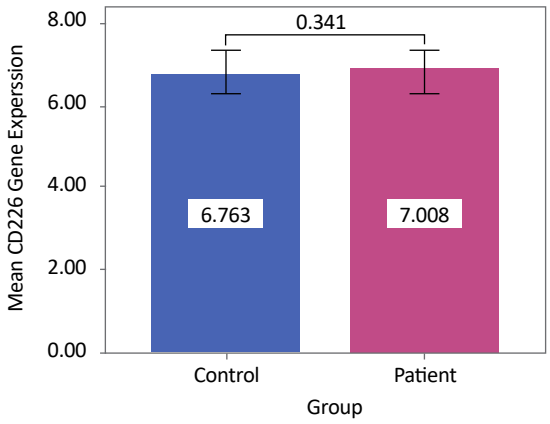

Figure 1. The relative expression of *CD226* in peripheral blood of the patients and the controls

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Determination of *CD226* serum level

Blood samples (3 mL) were collected from the patients with MS and the controls, using serum-separating tubes. The blood samples were centrifuged at 1500×g for 4 min and the cell-free sera were stored at -80ºC for the Enzyme-Linked Immunosorbent Assay (ELISA). The soluble *CD226* levels were measured in duplicate wells using human *CD226* ELISA kit (Eastbiopharm Cat. No: CK-E91994) according to the manufacturer's instructions. The standard curve was drawn using the derived values based on data from the ELISA reader (Hiperion, Germany). Optical Density (OD) at 450 nm wavelength was recorded in the blood samples of both groups. The sensitivity was 2.4 ng/L.

Statistical analysis

The Shapiro-Wilk test was used to study the normality of the data. The data were then analyzed by the Mann-Whitney U test. For all evaluations, $P<0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS.

Results

Levels of *CD226* gene expression

The mean number of *CD226* mRNA expressing blood was 7 in MS and was 6.76 in healthy controls. We compared *CD226* gene expression between healthy controls and new cases of MS in which none of the patients of this group experienced relapse during recent 3 months and were not on corticosteroid agents since at least recent 3 months. As shown in Figure 1, eventhough *CD226* expression increased in the patients, the difference was not statistically significant (P=0.341).

Table 3. Comparison of the serum levels of *CD226* protein (ng/L) in both studied groups

Marker	Group	No.	Mean±SD	SEM	P
Serum levels of *CD226* protein	Controls	30	2.18±2.04	0.40	0.978
	Patients	30	2.19±2.20	0.40	

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CD226 serum level

Soluble *CD226* was detected in the serum of all patients with MS and healthy subjects. The mean values of *CD226* serum level were 2.19 ng/mL in the case group and 2.18 ng/ in the control group. We did not detect any significant difference of *CD226* serum levels in the MS and control groups (P=0.978) (Table 3). The assessment of protein expression by ELISA confirmed the result of real-time technique. There was a correlation between the numbers of *CD226* mRNA expressing in the whole blood and serum levels of soluble *CD226* (P<0.05).

Discussion

Multiple Sclerosis is an autoimmune and neurodegenerative disease of the CNS. *CD226* is involved in the upregulation of Th1 and Th17 cells. Using flow cytometry for recognizing the exact cells that express *CD226* is important, because Th1 and Th17 cells play the most important role in pathogenesis of MS [28]. Here, we investigated expression of *CD226* in RRMS.

Our results showed no statistically significant difference in the level of *CD226* gene expression in peripheral blood sample of new cases of RRMS patients and healthy individuals. In contrast to our data, Gross et al. demonstrated that *CD226* expression reduced in MS patients. They also concluded that the higher threshold for NK-cell activation is attributable to the reduced *CD226* expression [29].

Another study also showed the association of non-synonymous exchange (Gly307Ser) in the gene for *CD226* variant with SSc and Wegener's Granulomatosis (WG) also demonstrated that (Single Nucleotide Polymorphism) SNPs located at *CD226* gene, such as rs727088 and rs763361, can influence *CD226* mRNA levels and different variant of these allels can be protective or predispose to autoimmune diseases [30, 33]. The SNPs of each person along with the gene expression of *CD226* are necessary for the exact conclusion [34].

The data regarding gene expression showed no significant changes in *CD226* protein expression levels. Since the coinhibitory receptor T cell Ig and ITIM domain (TIGIT) and the costimulatory factor *CD226* bind to the common ligand CD155 and the TIGIT transduce inhibitory signals and compete with *CD226* for binding with CD155 on the surface of antigen presenting cells [35, 36]. It also seems necessary to demonstrate further research on the gene expression of TIGIT.

The lack of association in this study also may be due to sample size or the course of MS disease, as others considered a greater sample size in their studies. Extensive variation in the prevalence of MS in different geographical districts and races may also contributed to the difference in the result of this study compared to other studies.

Conclusion

We determined no significant expression change of *CD226* in the blood samples of patients with MS. Therefore, measuring the expression of *CD226* gene is questionable as a biomarker for the diagnosis or improvement of MS. Since *CD226* targeting would exclusively target proinflammatory Th1 and Th17 cells, and these cells have the most important role in the pathogenesis of MS [28], additional studies are therefore required to measure *CD226* expression in certain subsets.

Ethical Considerations

Compliance with ethical guidelines

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee. This study was approved by the Ethics Committee of Isfahan University of Medical Sciences (Grant No: 396067).

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Conflict of interest

The authors certify that they have no affiliation with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials dismissed in this manuscript.

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