

Investigating the Extent of CCL4 and CCL5 Chemokine as Well as IL17 and IL23 Cytokine Gene Expression in the Patients Afflicted with Multiple Sclerosis

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Abstract

MS is a chronic inflammatory disease of central nervous system in which T cells enter central nervous system and create an inflammatory cascade that leads to applying the other blood cells. Pre-inflammatory Cytokines are representative of the process of inflammatory diseases like MS. In addition, the increase of such cytokines has been observed in this kind of anomalies. On the other hand, helping T cells of 17 (TH17) contributes to the secretion of interleukin 17 that is a pre-inflammatory cytokine and an increase in TH17 cells is induced by interleukin 23 (IL23) which in turn causes absorption of neutrophils in the site of inflammation. Chemokine also has a determining function in the immune system including Ccl4 protein sit, the function of which is chemical absorption for calling natural fatal cells and monocytes as well as the other immune system cells; additionally, Ccl5 causes chemical absorption to absorb Eosinophil and Basophil and has an active role in applying Leukocyte-sin the site of inflammation. As a result, these 4 factors can hurt nervous cell through increasing its expression and calling leukocytes. The expression of these 4 genes, corticosteroid treatment and investigating its impact on changing the expression of such genes are the aims of this study. In this study 50 samples of blood of people suffer from multiple sclerosis (according to the diagnosis of specialist) (new case), 25 samples of blood of people suffer from MS (according to the diagnosis of specialist) using corticosteroid and 50 blood samples of healthy people without any symptoms were compared. To investigate the extent of expression of IL23, IL-17, CCL5 and CCL4 genes, first, the patients' blood is taken, RNA was extracted and Real Time PCR was carried out. According to the results obtained from Real Time PCR of patients and healthy people it was specified that the amount of expression of chemokine and cytokines is increased in the people suffering from MS and has a direct relationship

with MS. In comparing the two groups of patients (new case and people taking medicine) it was revealed that the amount of expression of IL17, IL23, CCL4 and CCL5 genes is decreased in people who take medicine. An increase in the expression of IL17, IL23, CCL4 and CCL5 genes is the symptom of MS disease and contributes to its creation and progression. The difference in the expression of chemokine and cytokine genes can be used as an identifying maker in this disease. On the other hand, corticosteroid medicine can have a determining role in the increase of expression as well as the destructive function of immune system.

Keywords

Multiple Sclerosis, Chemokine, Cytokines, Real Time PCR

1. Introduction

Multiple sclerosis or MS is a chronic inflammatory disease of central nervous system which is known as an autoimmune disease. Exhaustion is one of the most important disabilities of this disease and the extent of outbreak is about 53 - 92 percent changeable depending on how to define exhaustion [1]. The exhaustion of this disease as the patients say is different from a regular one; it is along with intense weakness, leak of energy and feeling of disability. Pathogenesis of this disease is still unidentified, but generally a set of mechanisms involved in its pathogenesis are recommended including: A change in neurotransmitters, cessation in the continuous transmission of unmyelinated axons, transmission in the paths of arousal, wakefulness and alertness as well as the presence of especial amino acids, none of which are finally verified [2]-[4]. In fact, MS is known as an immunopathological disease in which T cells enter central nervous system and create an inflammatory cascade that leads to applying the other blood cells [5] [6]. However, migration of immune cells can be considered a potential cause of treatment [7]. Statistics all around the world show that women are afflicted with MS more than men, and generally 2 - 3 million Europeans and countries with Caucasian immigrants (such as America, Australia and north of Asia) are suffering from this disease [8] [9]. However, in a study the effect of geographical environment was investigated in single-egg twins by keeping them separated before puberty [10]. This disease like the other multifactorial diseases is influenced by genetic and environmental factors. Therefore, in the absence of etiological factors to be considered as the aim of prevention and cure, molecular mechanisms that are the foundation of inflammations, myelin omission and detoxification in neurons are used; this resulted in some ways of treatment and decreased progression of the disease, but it wasn't treated. Proper treatments of MS must prevent return of the disease, nervous inflammation, myelin omission and loss of neurons. But, unfortunately there is no treatment like this and treatment can reduce progression of the disease through anti-inflammatory glucocorticosteroids. Prescription of INF copolymer and the recent use of new pharmaceuticals of natalizomab and antibody monoclonal (Moab) against $\alpha 4 \beta 1$ integrin can enhance such people's life

expectancy and quality of life. However, limitations of treatment as well as the side effects of such pharmaceuticals made scientists have innovations in treatments [11]. Pre-inflammatory cytokines that are representative of inflammatory process of diseases such as MS are associated with the symptoms and exhaustion of many diseases like cancer, viral infection and exhaustion syndrome [12]-[15]. Besides, the increase of cytokines especially the increase of alpha tumor necrosis factor (TNF α) has been observed in such anomalies and caused sleepiness during the day, sudden falling asleep, sleep attack and idiopathic oversleeping [16] [17]. 17 helper T cells contribute to the secretion of interleukin 17 that is a pre-inflammatory cytokine; the increase of TH17 cells is induced by interleukin 23 and interleukin itself causes absorption of neutrophil to the site of inflammation and affects creation of inflammation [18] [19]. So, the role of such cytokines is very significant in the pathology of MS disease. On the other hand, chemokine is the factor that calls immune cells to migrate and the probability of inflammatory diseases by targeting the chemokine and its receptors have led to many researches. Chemokine is hardly fallen into two groups: First, pre-inflammatory chemokine that calls leukocytes to the site of inflammation when need be and second, homeostatic or anti-inflammatory chemokine that are regulators of continuous transmission to the site of main lymphoid. Previous studies generally focused on inflammatory chemokine. Most of the anomalies of MS disease are due to the function of B cells and patients manifest production of intrathecal immunoglobulin that has an oligo colony-band and responds specifically. The immunoglobulin oligo colony will remain in patients for a long time [20]. In the laboratory it is specified that the speed of transmission of B cells in the brain endothelium is more than T cells [21]. But, it is not specified how the chemokine that leads B cells is regulated [22]. Ccl4 or ligand chemokine is a human protein encoded by a gene under the same name, another name of which is beta MIP1 is responsible for chemical absorption for calling natural fatal cells, monocytes and the other cells of immune system [23]. Ccl5 human protein set which is copied by a gene under the same name causes chemical absorption in order to absorb eosinophil and basophil and has a determining role in applying leucocytes in the site of inflammation [24]. That's why such chemokine is essential in calling defensive cells to the site of inflammation, therefore, the change of their expression is very important in the body of patient, and thus the chemokine mentioned in the other studies were chosen. At the end, it can be stated that we will be able to diagnose patients faster and use promising treatments for them by applying new tools such as sequencing the entire genome and the related studies [25] [26] as well as investigating expressive profile and protein analysis [27] [28].

The aim of this study is to investigate the extent of change in the expression of 4 genes including 2 cytokines of IL17 and IL23 as well as 2 chemokine CCL4 and CCL5 in the patients afflicted with MS compared to healthy people, because the outcome of the 4 aforementioned genes have determining role in stimulating immune system and moving it toward inflamed nervous cells and in case of an increase in the expression, they will be the aim of treatment in the subsequent studies.

2. Materials and Methods

Molecular investigation of chemokine and cytokine genes using Real Time PCR method

Real Time PCR was used to investigate the extent of expression of IL23, IL-17, CCL4 and CCL5 genes. In this study 50 blood sample of people afflicted with Multiple Sclerosis (according to the diagnosis of specialist) was taken from Shohada Tajrish hospital (new case), 25 blood sample of people afflicted with Multiple Sclerosis (according to the diagnosis of specialist) who used corticosteroid was taken from Shohada Tajrish hospital and 50 sample of healthy people without any symptoms were compared. First, RNA was extracted with QIAamp RNA Blood Mini Kits (Qiagen, Germany) and the quality of each of them was assessed using Nanodrop. All the samples enjoy good quality and thickness and they are in the range of 1.8 - 2 (**Table 1**, **Table 2**).

Perl primer (version 20) and primer 3 software were used for primary designing of the required genome, after a proper pair of primers were selected with regard to the mentioned points, their connection was investigated on the genome through Ncbi BLAST site (National Center for Biotechnology Information) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Synthesis of cDNA

The synthesis was carried out using cDNA synthesis kit (Vivantis America) following the guidelines of the manufacturing company by adding Rnase inhibitor for removing cDNA pollution of synthesis in 5 minutes in 65 degree centigrade.

CDNA that was made using PCR method obtained proliferation, temperature and cDNA dilution and after electrophorus gel and making sure of the correction, Real Time PCR was used.

Table 1. Primers and the sequence of probes.

Gene	Primers and the sequence of probes
Beta actin (β -actin)	Forward 5-AGCCTCGCCTTTGCCGA-3 Reverse 5-CTGGTGCCTGGGGCG-3
CCL4	Forward 5-TCCTACTGCCTGCTGCTT-3 Reverse 5-GCTGGTCTCAAAGTAATCTGC-3
CCL5	Forward 5-TTCTACACCAGCAGCAAG-3 Reverse 5-TTCTACACCAGCAGCAAG-3

Table 2. Primers and the sequence of probes.

Gene	Primers and the sequence of probes
Beta actin (β -actin)	Forward 5-AGCCTCGCCTTTGCCGA-3 Reverse 5-CTGGTGCCTGGGGCG-3
Interleukin-17 (IL-17A)	Forward 5-AATCTCCACCAGCAATGAGGA-3 Reverse 5-ACGTTCCCATCAGCGTTGA-3
Interleukin{IL-23 (p19)}	Forward 5-TCAGTGCCAGCAGCTTTCAC-3 Reverse 5-TCTCTTAGATCCATGTGTCCAC-3

Real Time PCR:

After extraction of cDNA of beta actin sample as control and IL17, IL23, CCL4 and CCL5 genes were investigated for determining the percent of changes in gene expression using Real Time PCR (Polymerase Chain Reaction). Therefore, investigating the extent of expression of IL17, IL23, CCL4 and CCL5 compared to beta actin gene (control homey gene) in the same samples was estimated in the form of relative expression.

Real Time PCR reaction was carried out using Master Mix 2x (Biorad America) in final volume of 20 microliter by qiagene Real Time PCR and the data of cycle of threshold was analyzed through System software ver.2.0. For estimating the percent of changes (Fold change) in the expression of IL17, IL23, CCL4 and CCL5 genes $2^{-\Delta\Delta CT}$ formula was used in which $\Delta\Delta CT$ of the difference in the cycle of threshold of IL17, IL23, CCL4 and CCL5 genes compared to the control gene in the sample was determined. Eventually, for estimating $2^{-\Delta\Delta CT}$, excel 2013 and SPSS software were used.

In the first real time we set up the required genes so that real time is carried out under the best conditions and dilution and after that we investigate the required samples under the same conditions.

Findings

We measured and investigated the prepared cDNA using Real-time PCR, Q model from QIAGEN Company.

The following picture (**Figure 1**) shows an increase in the expression of ccl4 and ccl5 genes in patients compared to healthy people:

Figure 2 shows an increase in the expression of IL17 and IL23 genes in the patients compared to healthy people.

Figure 3 shows the difference of expression of ccl4 and ccl5 in the patients who used corticosteroid and the healthy people.

Figure 4 shows the difference of expression of IL17 and IL23 in the patients who used corticosteroid and healthy people.

The results of Real Time reveals an increase in the expression of IL17, IL23, CCL4 and CCL5 genes in the patients suffering from multiple sclerosis, such genes are higher expression in this disease. Corticosteroid treated patients had an increase in the expression of IL17, IL23, CCL4 and CCL5 genes, however this increase is not to the extent of new case people.

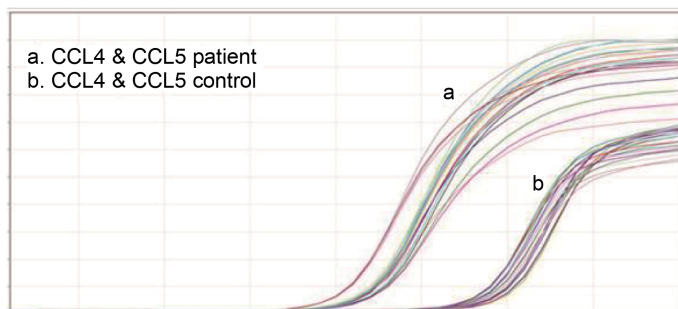


Figure 1. Increase in the expression of ccl4 and ccl5 genes in patients compared to healthy people.

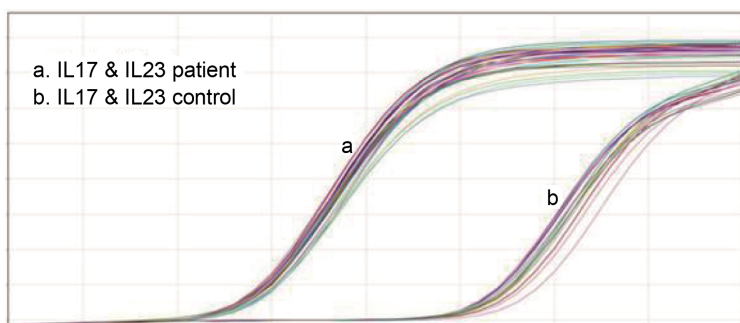


Figure 2. Increase in the expression of IL17 and IL23 genes in the patients compared to healthy people.

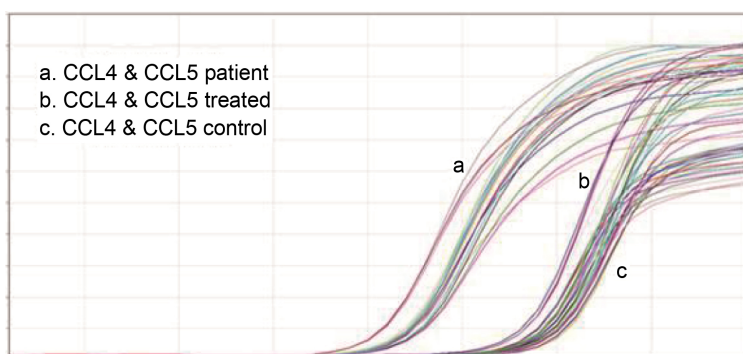


Figure 3. Difference of expression of ccl4 and ccl5 in the patients who used corticosteroid and the healthy people.

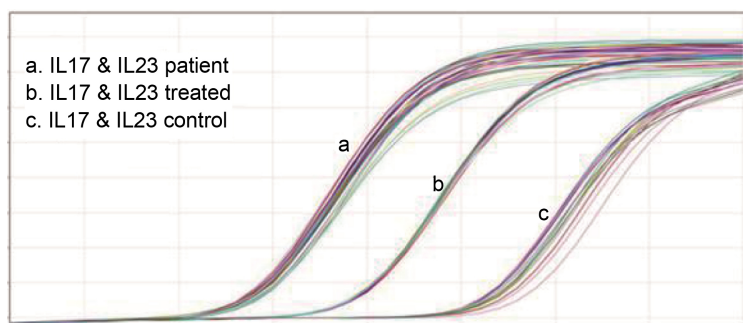


Figure 4. Difference of expression of IL17 and IL23 in the patients who used corticosteroid and healthy people.

3. Discussion

We know that keeping dynamic balanced homeostasis among the pre-inflammatory and anti-inflammatory cytokines is required. Pre-inflammatory cytokines have a key role in MS pathogenesis through activating immune system in blood and central nervous system. Anti-inflammatory cytokines containing IL-4 are effective in the recovery of MS. Pre-inflammatory cytokines include: IFN-gamma, TNF-beta, IL-12 and IL-17, the blood cells expressing mRNA, the number of TNF α and its serum concentration are increased in MS patients. IL-12, the most stimulant agent of IFN-gamma is also a

pre-inflammatory cytokine, but the new information reveal that IL-23 whose P40 chain is similar to IL-12 has more important role. Pre-inflammatory cytokines cause Oligodendrocyte and myelin harm to CSF and brain. More mononuclear cells expressing TNF α and IFN-gamma are seen in MS patients. TNF α is a pre-inflammatory cytokine and makes inflammation, but it also contributes to tissue repair in brain. Pre-inflammatory cytokines of IL-12 and IL-17 are also increased in CSF and brain lesions of MS patients [29]-[32]. Contributive 17 T cells contribute to the secretion of IL17 that is a pre-inflammatory cytokine, the increase of TH17 cells is induced by IL23 and IL17 itself causes absorption of neutrophils to the site of inflammation and create inflammation [18] [19]. Chemokine and its receptors also have a key role in calling leukocytes and the other cells to the site of inflammation. Entering inflammatory T cells inside CNS is the most important step in MS. Blood-cerebral blockage will be started by weak connection of these cells with endothelial cells. Then, they make a firm cellular connection and pass blood-cerebral blockage. Chemokine activate the connection of leukocyte cells with endothelial and this way they pass blood-cerebral blockage. Induction of proteolytic enzymes removes blood-cerebral blockage and chemokine keep entrance of the other cells to CNS. The expression of CCR5 in T cells is increased in the environmental blood of MS patients, besides, in recurrence which is representative of pathogenic role for +CTL CCR5 cells, the increase of expression of CXCR3 was observed in the T cells of some of the patients. T cells expressing CCR5 and CXCR3 produce high amount of TNF α and IFN-gamma and specific Th1 cells MBP express high level of CXCR3 and CXCR6. The expression of CXCR3 facilitates the entrance of T cells to CNS and CXCL10 keeps such cells in inflamed CNS. CCL4 is activated in parenchyma inflammatory cells (macrophage and microglia), CCL3 is activated in inflammatory cells of parenchyma and neuroglia and CCL5 is expressed in the inflammatory cells of pre-vessel and astrocytes. The other chemokine in active lesions of MS include: CCL2, CCL7, CCL8 and CXCL10. CXCR3 is expressed over most of the pre-vessel T cells of MS lesion and CCR5 is expressed over some of these cells.

CCR1 is expressed on the newly infiltrated monocytes, CCR2 and CCR3 are expressed on macrophage and CCR5 is expressed on the infiltrating monocytes and activated microglia cells. The role of chemokine and its receptors was verified in MS pathogenesis through a research on the animal model (EAE) of MS. The increase of CCL3, CCL2, CCL5 and CXCL10 in EAE is along with the progression of disease and their elimination results in recovery. Polymorphism in chemokine genes and its receptors cause sensitivity or immunity to MS, however, there is no certain evidence to confirm this hypothesis [29]. Ccl4 or chemokine ligand is a human protein being encoded by a gene under the same name and another name of that is MIP1. Its function is chemical absorption to call natural fatal cells, monocytes and the other immune system cells [23] [33]. Ccl5 is a human protein which is copied by a gene in the same name, this protein causes chemical absorption to absorb eosinophil and basophils and it has an active role in applying leucocytes in the site of inflammation [24]. The aim of this research is to investigate the impact of MS disease on production of cytokine and che-

mokine by the immune system of the patient. The results were the ones predicted according to the rule and the level of all 4 factors had an increase in expression, this represented that the patient had inflammatory reaction and the cytokines and chemokine are activated to call the other parts of immune system and they apply the other factors. In this study corticosteroid treatment was applied to test and prove its function. Using it for a period, the level of expression of these 4 genes were investigated. The result revealed that it decreased their expression influentially and it reduced inflammation.

4. Conclusion

According to the data it was specified that the 4 genes had an increase in expression and the increase of their expression leads to calling leucocytes and nervous harm; therefore, this increases and subsequently calling leucocytes to the site of nervous cells are harmful for cell and result in some harms that create disease, so however we can reduce the increase in expression, and the life expectancy and the analysis of high level of symptoms will be increased. According to the study, it can be stated that corticosteroid can be a reliable pharmaceutical, but the side effects must also be taken into account. It is recommended to investigate the lower factors of such genes as well and it is better to try herbal-based pharmaceuticals.

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