J Mol Neurosci (2015) 55:891–897
DOI 10.1007/s12031-014-0443-z

Circulating Levels of Interleukin-35 in Patients with Multiple Sclerosis: Evaluation of the Influences of FOXP3 Gene Polymorphism and Treatment Program

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Received: 5 September 2014 / Accepted: 10 October 2014 / Published online: 19 October 2014

Abstract The regulatory T (Treg) cells play a major role in the control of the autoimmunity and inflammation, and IL-35 has been described as an immunosuppressive cytokine that is mainly produced by $CD4^+$ $FOXP3^+$ Treg cells. The aim of this study was to evaluate the serum levels of IL-35 and a single nucleotide polymorphism (SNP), rs3761548, in *FOXP3* gene in patients with multiple sclerosis. The blood samples were collected from 140 multiple sclerosis (MS) patients (including 51 untreated and 89 treated patients) and 140 healthy subjects as a control group. The serum levels of IL-35 were measured by ELISA. The DNA was analyzed for SNP rs3761548 in *FOXP3* gene using SSP-PCR. There was no significant difference between untreated MS patients and control group regarding the mean serum levels of IL-35, although this parameter was higher in untreated patients. However, the mean serum level of IL-35 in treated MS patients was significantly higher than that in the control group ($P<0.008$). The mean serum levels of IL-35 in patients who were treated with interferon-β, methylprednisolone, or with the both interferon-β and methylprednisolone were significantly higher than that in the healthy group ($P<0.01$, $P<0.01$, and $P<0.2$, respectively). The frequencies of AA and AC genotypes at rs3761548 in the *FOXP3* gene were significantly higher in MS group as compared with healthy subjects ($P<0.05$). The frequency of CC genotype at rs3761548 was significantly lower in the MS group in comparison with healthy control subjects ($P<0.001$). Moreover, the frequency of A allele was significantly higher whereas the frequency of C allele was significantly lower in MS patients in comparison to healthy subjects ($P<0.001$). The mean serum level of IL-35 was significantly lower in MS patients or healthy subjects with AA genotype as compared with those with CC genotype at rs3761548 in *FOXP3* gene ($P<0.01$ and $P<0.001$, respectively). These results showed higher serum levels of IL-35 in treated MS patients representing that the benefit effects of treatment may in part performed through the upregulation of the IL-35 production. The SNP rs3761548 may influence the susceptibility to MS disease and the serum levels of IL-35.

Keywords Multiple sclerosis · IL-35 · FOXP3 · Gene polymorphism · Treatment

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) resulting from an abnormal immune response against myelin antigens (Koziol et al. 2005). The immune system plays an important role in the pathogenesis and complications of MS disease (Graber et al. 2010). T-helper (Th) cell-dependent immune

responses, in particular, perform a key role in the pathogenesis of MS disease (Buc 2013a). Upon antigenic stimulation, the T $CD4^+$ cells differentiate into several subsets such as Th1, Th2, Th17, and regulatory T (Treg) cells which is characterized by distinct cytokine profile (Hirahara et al. 2013; Yamane and Paul 2013). The Th1-related cytokines (such as IFN-γ and TNF-α) and Th17-related cytokines (IL-17 and IL-22) lead to MS disease progression and worsening of symptoms whereas the Th2-related cytokines (such as IL-4, IL-10, and IL-5) and Treg-related cytokines (TGF-β and IL-10) have been associated with the reduction and improvement of symptoms in MS patients (Buc 2013b; Tumani et al. 2011; Wang et al. 2013). It has been demonstrated that MS is a Th1/Th17-mediated autoimmune disease (Aranami and Yamamura 2008).

Immunologically, Treg cells comprise a subset of $CD4^+$ lymphocytes that play a major role in regulating the immune responses by their ability to suppress Th cells, and their defects may lead to the development of autoimmune diseases (Singer et al. 2014). The natural Treg (nTreg) cells are originated from the thymus and comprise 5–10 % of the circulating $CD4^+$ T cell population (Dasgupta and Saxena 2012). Induced Treg (iTreg) cells were differentiated in the periphery following antigenic stimulation in the presence of cytokines such as IL-2 and TGF-β. The iTreg cells are more divided into subsets based on the mechanisms that they mediate immunoregulation including Tr1 cells that mediate suppression primarily through the secretion of the IL-10 and are characterized by the lack of the FOXP3 and CD25 expression which are expressed by nTreg cells (Peterson 2012). The other class of iTreg cells was named as Th3 cells that express CD25 and FOXP3 and mainly utilize TGF-β to mediate suppression (Peterson 2012). However, the Treg cell-related immunosuppressive cytokines were identified as IL-10, TGF-β, and IL-35 (Gravano and Vignali 2012).

IL-35 is an immunosuppressive cytokine and is composed of P35 and Epstein-Barr virus-induced gene 3 (EBI3) subunits, which is expressed primarily by Treg cells (Belladonna and Grohmann 2013; Olson et al. 2013). The expression of IL-35 has been also identified in a population of $CD4^+$ Treg cells, defined as iTr35 cells (Olson et al. 2013). IL-35 acts on its target cells following binding to the IL-35 receptor. The IL-35 receptor is composed of IL-12Rβ2 and gp130, which are also related with the IL-12 and IL-27 receptors, respectively (Ye et al. 2013). Following binding of IL-35 to its receptor, it signals through the STAT1 and STAT4, which result to the expression of target genes including P35 and EbI3, resulting in a feedback loop promoting more IL-35 expression. Accordingly, IL-35 and Treg cells reciprocally upregulate each other (Olson et al. 2013; Ye et al. 2013). More recently, it has been reported that IL-35, rather than IL-10 or TGF-β, was required for Treg-mediated suppression (Olson et al. 2013).

It has been also demonstrated that IL-35 plays a role in the suppression of Th1 and Th17 responses. The Treg cells expressing IL-35 have been shown to inhibit the differentiation of $CD4^+$ T cells into Th17 effector cells. The mice lacking EBI3 produce higher levels of IL-17 (Olson et al. 2013). IL-35 also suppresses the antibody responses (Olson et al. 2013). The defects in the IL-35 production have been associated with the development and aggravation of a number of inflammatory diseases such as encephalomyelitis, inflammatory bowel disease, liver fibrosis, and models of lethal autoimmune disease (Olson et al. 2013). The induction of IL-35 production has been shown to ameliorate the disease symptoms in the experimental colitis and collagen-induced arthritis (Belladonna and Grohmann 2014; Olson et al. 2013). IL-35 can also induce infectious tolerance and may increase tumor progression (Olson et al. 2013). Furthermore, allergic airway inflammation was ameliorated by IL-35 through the suppression of circulating specific and total IgE (Bai et al. 2012).

It has been reported that the fork head box protein 3 $(FOXP3)^+$ Treg cells could produce IL-35 (Collison et al. 2012). The FOXP3 is a central molecule in the function of Treg cells that play an important role in the immunoregulation (Katoh et al. 2013). It has also been demonstrated that the continued expression of FOXP3 in mature Treg cells is essential for the maintenance of the Treg cell-mediated tolerance (Oda et al. 2013). The gene coding for this transcription factor is located on chromosome X. Mutations in *FOXP3* gene in humans cause immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) which is characterized by a high incidence of autoimmune diseases (Katoh et al. 2013). Moreover, polymorphisms in *FOXP3* gene can lead to immune system irregularities and mediate the development of the autoimmune disease (Oda et al. 2013). Several polymorphisms have been reported in the promoter, intron, and exon regions of the *FOXP3* gene (Oda et al. 2013). The promoter involves in the initiation of the transcription and is therefore among the major elements that control gene expression (Oda et al. 2013). The polymorphisms in the promoter region may potentially alter the gene expression by changing the binding of transcription factors to their binding sites and by changing the kinetics of the transcription process. The SNP −3279C/A (rs3761548) is among of the polymorphisms located in the promoter region of *FOXP3* gene (He et al. 2013; Oda et al. 2013). However, the association of the SNP −3279C/A (rs3761548) with MS has not been investigated, yet.

Investigations of the peripheral blood have implicated an important role for Treg cells in the pathogenesis of MS. The defects in the function, but not frequency of $CD4^+CD25^+$ Treg populations, have been reported in MS patients (Buc 2013c). Moreover, the functional impairments of the Treg cells have been associated with lower FOXP3 expression (Buc 2013c). To our knowledge, the serum concentration of IL-35 has not yet been evaluated in the patients with MS. Accordingly, this study was conducted to investigate the serum levels of IL-35

and the SNP −3279C/A (rs3761548) in *FOXP3* gene in patients with MS disease to clarify any association.

Material and Methods

Subjects

Peripheral blood samples were collected from 140 patients with MS disease (46 men and 94 women) from January 2013 to February 2014 in the Shephah Hospital of Kerman (a city located in the southeast of Iran). The expert neurologists confirmed the existence of MS disease, on the basis of the clinical and paraclinical examinations according to the McDonald's criteria (McDonald et al. 2001). The patients have RRMS (n=102), SPMS (n=28), PPMS (n=8) and PRMS (n=2) patterns of the disease. According to the diagnostic time and treatment, the patients were classified as newly diagnosed (untreated) patients (n=51) if they did not receive any drug and treated patients (n=89) if they were treated with methylprednisolone, interferon-β, or both interferon-β and methylprednisolone (Table 1).

In a total, 140 healthy subjects (47 men and 93 women) were also enrolled into the investigation as a control group. The healthy subjects were recruited among blood donations of the Kerman Transfusion Organization and interviewed regarding CNS disease, and none of them had any history of CNS diseases or other relevant disorders. All control subjects were basically healthy, with no acute or chronic sickness. Indeed, persons with disease (such as history of recurrent infections, asthma, allergy, and atopic diseases; any suspected immunological disorders; cigarette smoking; and use of any drugs) were all excluded from the investigation. The other exclusion criteria were malignancy, surgery, and major trauma within 6 months prior to the blood collection.

Table 1 Serum levels of IL-35 in newly untreated and treated MS patients according to gender

Group	Sex	Number	IL-35 levels (mean±SD)[a]	P value
Newly diagnosed MS patients	Male	25	4.09±1.67	0.12
	Female	26	1.63±0.14	
	Total	51	2.78±0.79	
Treated MS patients	Male	21	7.35±2.35	0.36
	Female	68	4.80±1.54	
	Total	89	5.60±1.29	
Healthy	Male	47	2.99±1.11	0.09
	Female	93	1.51±0.18	
	Total	140	1.99±0.38	

[a] The serum levels of cytokine expressed as Pg/ml

This investigation was evaluated and approved by the Ethical Committee of Kerman University of Medical Sciences. Moreover, patients were recruited if they agreed for blood sampling. A peripheral blood sample (2–4 ml) was collected from all participants, and the sera were separated and stored at −70 °C until analysis.

DNA Extraction

The peripheral blood was collected in EDTA pre-coated tubes and then the genomic DNA was extracted from peripheral blood leukocytes by salting out method as previously described by Miller et al. (Miller et al. 1988). Extracted DNA samples were stored at −20 °C until analysis.

Polymorphism Genotyping

The *FOXP3* gene polymorphism at SNP rs3761548 was determined by the polymerase chain reaction–sequence-specific primer (PCR–SSP) technique. The reaction mixture of the PCR was made up by the addition of the following reagents to a 0.2-ml microcentrifuge tube on ice: 2.5 μl of Taq DNA polymerase buffer (10×), 0.5 μl of MgCl2 (stock concentration 1.5 mM), 0.5 μl of each dNTP [dATP, dCTP, dGTP, and dTTP (stock concentration of 10 mM)], 1 μl of each primer [forward primers: 5-CTGGCTCTCTCCCCAACTGA-3 and 5-TGGCTCTCTCCCCAACTGG-3 and common reverse primer: 5-CAGAGCCCATCATCAGACTCTCTA-3 (stock concentration of 25 ng/μl)], 1 μl of prepared DNA, and sterile double-distilled water to a final volume of 25 μl. The amplification was performed with the following program:

One initial stage of 94 °C for 4 min, followed by 30 cycles of 94 °C for 40 s, 61 °C for 30 s, and 72 °C for 40 s. The amplified PCR product of *FOXP3* gene covers the SNP rs3761548 with a molecular size of 334 or 333 bp in the presence of A allele or C allele, respectively. The products were electrophoresed on a 2.5 % agarose gel after adding 4 μl of loading buffer (Cinnagen, Iran) and studied on a Chemi-Doc model XRS (Bio-Rad, USA) after staining with ethidium bromide.

Determination of the IL-35 Concentrations

The serum levels of IL-35 were measured by commercial ELISA kits (Biospes systems, China) according to the manufacturer's guidelines. The sensitivity of the assay was 3 Pg/ml.

Statistical Analysis

Differences in the variables were analyzed by using Student's t test, ANOVA, and $\chi 2$ tests as appropriate, and the P values of less than 0.05 were considered significant. Hardy–Weinberg equilibrium was assessed using genotype data. The allele

and genotype frequencies were calculated in patients and healthy controls by direct gene counting. The data were analyzed by statistical SPSS software (version 18, Chicago, IL, USA).

Results

The mean ages of MS patients and the healthy control group were 34.98±8.25 years and 36.07±8.11 years, respectively (P =0.26). The gender proportions of patients were 94 (67.1 %) for women and 46 (32.9 %) for men and for healthy control group was 93 (66.4 %) for women and 47 (33.6 %) for men (P =0.50).

The Serum Levels of IL-35 in Treated and Untreated MS Patients

The mean serum levels of IL-35 in untreated and in treated MS patients according to their gender have been demonstrated in Table 1. There was no significant difference between the untreated MS patients and healthy control group regarding the mean serum levels of IL-35, although this parameter was higher in untreated patients. However, the mean serum level of IL-35 in treated MS patients was significantly higher than that in the healthy control group (P<0.008). In both treated and untreated patients and also in healthy control group, the mean serum levels of IL-35 were higher in men in comparison with women, but the differences were not statistically significant (Table 1).

The mean serum levels of IL-35 in treated men and women patients were significantly higher than those in healthy subjects with the same gender (P<0.01 and P<0.05, respectively). No significant differences were observed between men and women of untreated patients and healthy subjects with the same gender regarding the mean serum levels of IL-35.

The Serum Levels of IL-35 in MS Patients According to Treatment Program

The mean serum levels of IL-35 in treated MS patients according to the treatment have been demonstrated in Table 2. No significant differences were observed between untreated patients and patients who were treated with interferon-β (IFN-β), methylprednisolone, or with both IFN-β and methylprednisolone with respect to the serum levels of IL-35, although this parameter was higher in treated patient groups. The mean serum levels of IL-35 in patients who were treated with IFN-β, methylprednisolone, or with both IFN-β and methylprednisolone were significantly higher than that in the healthy group (P<0.01, P<0.01, and P<0.02, respectively).

Table 2 Serum levels of IL-35 MS patients according to treatment program

Groups	Number	IL-35 levels (mean±SD)[a]	P value
Interferon	6	7.84±6.78	0.03
Methylprednisolone	18	7.22±3.52	
Methylprednisolone + interferon	65	4.75±1.21	
No treatment	51	2.78±0.79	
Healthy controls	140	1.92±0.38	

[a] The serum levels of cytokine expressed as Pg/ml

Genetic Variations at SNP rs3761548 in the *FOXP3* Gene in MS Patients and Healthy Control Group

The frequencies of the genotypes and alleles at SNP rs3761548 in the *FOXP3* gene in MS patients and healthy control subjects have been summarized in Table 3. The frequencies of AA and AC genotypes at rs3761548 in the *FOXP3* gene were significantly higher in the MS group as compared with healthy control subjects (P<0.05). The frequency of CC genotype at rs3761548 in the *FOXP3* gene was significantly lower in the MS group in comparison with healthy control subjects (P<0.001). Moreover, the frequency of A allele was significantly higher whereas the frequency of C allele was significantly lower in MS patients in comparison to healthy subjects (P<0.001).

The Serum Levels of IL-35 According to Genetic Variations at SNP rs3761548 in *FOXP3* Gene

The serum levels of IL-35 in MS patients and healthy control group according to genetic variations at SNP rs3761548 in *FOXP3* gene have been demonstrated in Table 4. In both patients and healthy control groups, there was significant difference among subjects with CC, AC, and AA genotypes at rs3761548 in *FOXP3* gene regarding the mean serum levels of IL-35. The mean serum level of IL-35 was significantly higher in MS patients or healthy subjects with CC genotype as compared with those with AA genotype at rs3761548 in *FOXP3* gene (P<0.01 and P<0.001, respectively). Moreover,

Table 3 The frequencies of the genotypes and alleles at SNP rs3761548 in the FOXP3 gene in MS patients and healthy control subjects

Genotypes	MS patients' no. (%)	Healthy subjects' no. (%)	P value
AA	34 (24.3 %)	21 (15 %)	0.05
AC	50 (35.7 %)	34 (24.3 %)	0.05
CC	56 (40 %)	85 (60.7 %)	0.001
A	118 (42.1 %)	76 (27.1 %)	0.001
C	162 (57.8 %)	204 (72.8 %)	0.001

Table 4 The serum levels of IL-35 MS and healthy groups according to the genetic variations at SNP rs3761548 in FOXP3 gene

Group	Genotype	Number	IL-35 levels (mean±SD)[a]	*P* value
MS	AA	34 (24.3 %)	1.27±0.16	0.04
	AC	50 (35.7 %)	3.18±0.91	
	CC	56 (40 %)	6.18±1.50	
	A	118 (42.1 %)	2.75±0.71	0.09
	C	162 (57.8 %)	4.85±0.93	
Healthy	AA	21 (15 %)	0.93±0.11	0.01
	AC	34 (24.3 %)	1.19±0.18	
	CC	85 (60.7 %)	2.77±0.69	
	A	76 (27.1 %)	1.10±0.12	0.14
	C	204 (72.8 %)	2.19±0.45	
Total	AA	55 (19.6 %)	1.11±0.10	0.03
	AC	84 (30 %)	2.55±0.63	
	CC	141 (50.4 %)	4.85±0.96	
	A	194 (34.6 %)	2.17±0.47	0.06
	C	366 (65.3 %)	3.89±0.62	

[a] The serum levels of cytokine expressed as Pg/ml

the mean serum level of IL-35 was higher in both MS patients or healthy subjects with C allele as compared with those with A allele at rs3761548, but the differences were not statistically significant (Table 4).

Discussion

IL-35 identified as a Treg cell-related cytokine with potent inhibitory effects on the immune system that prevents the inflammatory responses and attenuates the autoimmune diseases (Ye et al. 2013). Although there are several investigations regarding the Treg cell abnormalities in MS patients (Buc 2013c; Lowther and Hafler 2012), however, the results of the present study showed that there was no significant difference between untreated MS patients and healthy control group regarding the serum levels of IL-35. These observations indicate that IL-35 may have no role in the initial development of MS disease.

Our results also showed that the mean serum levels of IL-35 in treated MS patients were significantly higher than that in the control group. Indeed, the serum levels of IL-35 in patients who were treated with IFN-β, methylprednisolone, or with both IFN-β and methylprednisolone were significantly higher than those in the healthy group. The IFN-β is the first biologic agent that has been used for the treatment of MS disease (Buc 2013c). Several immunomodulatory effects have been attributed to the IFN-β such as downregulation of the expression of HLA class II molecules and co-stimulatory molecules on the antigen-presenting cells; reduction of the production of inflammatory cytokines including IFN-γ, IL-12, and IL-17; upregulation of the expression of PDL-2; inhibition of the proliferation of macrophages and T cells; downregulation of the VLA-4 adhesion molecules; and inhibition of the matrix metaloproteinase (MMP-9) production by activated T cells which inhibit the rupture of the blood brain barrier (BBB) (Buc 2013c). Several immunomodulatory effects have been also attributed to methylprednisolone such as reduction of the CCL21, CXCL11, and IL-18 production (Rentzos et al. 2008; Szczucinski et al. 2007); upregulation of the IL-10 production (Rentzos et al. 2008); inhibition of the IFN-γ and IL-17 expression (Miljkovic et al. 2009); and downregulation of the expression of ICAM-1 and VCAM-1 adhesion molecules (Gelati et al. 2000).

As mentioned, the reduced suppressive functions of Treg cells have been demonstrated in MS patients (Buc 2013a; Lowther and Hafler 2012). It has been reported that both IFN-β and methylprednisolone influence the Treg cell activities. IFN-β increases the expression of the ligands for GITR on dendritic cells. The interaction between GITR on Treg cells and GITR ligands on dendritic cells induces the proliferation of Treg cells (Buc 2013a). It has also been demonstrated that IFN-β significantly increases the frequency of the $CD4^+CD25^+FOXP3^+$ Treg cells in MS patients (Namdar et al. 2010). The positive effects of methylprednisolone on the differentiation and maturation of Treg cells have also been demonstrated (Seissler et al. 2012). Our findings regarding the higher levels of IL-35 in treated patients represent that the benefit effects of IFN-β and methylprednisolone may in part perform through the upregulation of the Treg cell activity and therefore by the increasing IL-35 expression.

The results of the present study also showed that the frequencies of AA and AC genotypes or A allele at rs3761548 in the *FOXP3* gene were significantly higher in the MS group as compared with healthy subjects. Accordingly, the SNP rs3761548 in the *FOXP3* gene may have an association with susceptibility to MS disease. There is no previous study regarding the association of rs3761548 in the *FOXP3* gene with MS. However, consistent with our results, the association of the rs3761548 in the *FOXP3* gene with susceptibility to several autoimmune diseases including Graves' disease, Hashimoto's disease, Crohn's disease, primary biliary cirrhosis, myasthenia gravis, alopecia areata, psoriasis, and vitiligo has been reported (He et al. 2013).

It has also been suggested that SNP rs3761548 in the *FOXP3* gene may have similar effects in populations with different ethnicities, different lifestyles, and with genetic variations (He et al. 2013; Oda et al. 2013). The polymorphisms of *FOXP3* gene may change its role in functional or quantitative manners, therefore influencing the function of $CD4^+$ $CD25^+$ Treg cells (Oda et al. 2013). Several polymorphisms have been reported in the promoter, intron, and exon regions of the *FOXP3* gene (Oda et al. 2013). The polymorphisms in

the promoter region may potentially modify gene expression by changing the binding of transcription factors to their binding sites and by modifying the kinetics of transcription process. Therefore, the genetic polymorphisms in the promoter region of the of *FOXP3* gene have been widely studied in the context of autoimmune diseases. The SNP rs3761548 is among the polymorphisms located in the promoter region of *FOXP3* gene (He et al. 2013; Oda et al. 2013). Therefore, SNP rs3761548 may directly or indirectly alter the level of the FOXP3 protein expression in Treg cells.

It has been reported that the presence of AA genotype at SNP rs3761548 leads to the loss of binding of some transcription factors to the promoter and therefore leading to the defective transcription of FOXP3 (Oda et al. 2013). Accordingly, the presence of AA genotype or A allele at SNP rs3761548 may lead to a marked reduction of FOXP3 expression and therefore cause the defects in the Treg cell activity. In accordance with our findings, the results of a meta-analysis study showed that the presence of A allele at SNP rs3761548 in the *FOXP3* gene definitively contributes to autoimmune disease susceptibility. Interestingly, the results of this study also showed that the mean serum levels of the immunosuppressive cytokine, IL-35, was significantly lower in subjects with AA genotype as compared with those with CC genotype at rs3761548 in *FOXP3* gene.

In conclusion, the results of the present study showed higher serum levels of IL-35 in treated MS patients. The SNP rs3761548 in *FOXP3* gene may influence the susceptibility to MS disease. The serum levels of IL-35 may be affected by SNP rs3761548 in *FOXP3* gene and treatment by IFN-β and methylprednisolone.

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