INVESTIGATION OF CXCL10 (IP-10) AND CXCL12 (SDF-1) SERUM LEVEL VARIATIONS AND THEIR GENETIC VARIANT IN T1D PATIENTS: A STUDY IN IRANIAN TYPE-1 DIABETIC PATIENTS

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ABSTRACT

T1D is defined as a heterogeneous autoimmune disease. Chemokines are important factors in the pathogenesis of T1D. Therefore, current study aimed to examine the association between serum CXCL10 with its promoter polymorphism at position 1443 and CXCL12 and its known SDF-1 3’ A genetic variant in T1D patients. Blood samples were collected from 209 T1D patients and 189 healthy controls for DNA extraction. The extracted DNA was analyzed for CXCL10 and CXCL12 polymorphisms using PCR-RLFP. The serum CXCL10 and CXCL12 were measured by ELISA. The demographic data of the patients was collected by a questionnaire. Significant
difference between the A/A, A/G and G/G genotype and A and G alleles of polymorphisms at position +801 of CXCL12 observed in patients and controls. Both CXCL10 and CXCL12 were markedly elevated in T1D patients, but no association was observed between CXCL10/1443 promoter polymorphism in T1D patients and all of patient and controls showed GG genotype. Results of present study probably suggest that the serum CXCL10 and CXCL12 may play important role in T1D pathogenesis. Only the SDF-1 3’ A polymorphism is possibly important in pathogenesis of T1D while CXCL10 -1443 promoter polymorphism may not.

**Keywords:** Type 1 diabetes, CXCL10 (IP-10), CXCL12 (SDF-1), Polymorphism.

**INTRODUCTION**
Type 1 diabetes (T1D) is described as an organ-specific autoimmune disease, characterized by the destruction of the pancreatic beta cells. It is often associated with other autoimmune diseases including pernicious anemia, autoimmune thyroid disease (AITD), addison’s disease and rheumatoid arthritis [1]. Compelling evidences demonstrated that both genetic and environment are important in diabetes and both pro-inflammatory and anti-inflammatory cytokines and their related gene variants are pre-dominant in pathogenesis of this type of diabetes [2]. Therefore, diabetes is purposed as an immunological disorder with changed cytokine expression profile[2-4]. This autoimmune state arising from a complex interaction of immune, genetic and environmental factors [5-6]. Chemokines are known as cytokines that regulate the migration of lymphocytes and other peripheral blood leukocytes into the inflammatory sites, infection and injured tissues. In recent years their roles in the pathogenesis of several autoimmunediseases have been clarified [7]. Chemokines are low molecular weight proteins (8-17 kDa), which are classified into four subclasses as C, CC, CX3C and CXC. CXCL10 (IP-10) and CXCL12 (SDF-1) will fit in the CXC subclass of chemokines [8]. These chemokines play important roles in appropriate immune response and are amongst the main chemokines in recruitment of immune cells to the inflamed and infected organs [8–9]. We have previously demonstrated the increased levels of CXCL10 (IP-10) following hepatocytes isolation and primary culture. in response to different stimuli and pro-inflammatory cytokines (IFN-γ and TNF-α) treatment [10–11]. Recently the important regulatory role of CXCL12 (SDF-1) on other member of CXC subfamily in the
development of diabetes in NOD mice was established [12]. Furthermore, the CXCL12 (SDF-1) gene is located on chromosome 10q11.1 near type 1 diabetes susceptibility locus IDDM10, suggesting a contributory role for CXCL12 (SDF-1) to the induction of diabetes. Recent reports demonstrated that the SDF-1 3’ A variant is associated with the early onset of T1D in French population. The CXCL12 (SDF-1) and one of its corresponding receptors (e.g, CXCR4) are of high interest as therapeutic targets in different pathological situations including different types of cancer [13-14] AIDS (Patrussi and Baldari), systemic autoimmune and neuroinflammatory disorders (e.g MS, stroke, Alzheimer’s disease) [15]. Previous studies showed that the expression of CXCL12 (SDF-1) can be affected by its SDF-1 3’ A polymorphism at position +801 region [16], but there is not much reports on CXCL10 (IP-10) -1443 and its relation to CXCL10 (IP-10) circulating levels. Therefore, the aim of this study was to investigate the CXCL12 (SDF-1)/SDF-1 3’ A and CXCL10 (IP-10) -1443 polymorphisms, as well as CXCL10 (IP-10) and CXCL12 (SDF-1) circulating levels in Iranian T1D patients suffering from different clinical complications.

MATERIALS & METHODS

Subjects: Specimens were collected from 205 T1D patients and 189 healthy controls during 2008-2011 at Rafsanjan and Mashhad hospitals of Medical universities. Expert internists and pediatrician confirmed the occurrence of T1D, according to the clinical and paraclinical findings. Healthy controls were selected from Rafsanjan population with same sex, age and ethnic background, following approval by Rafsanjan University of Medical Sciences ethical committee. Written consent forms are filled out by both patients and controls prior to samples collection.

DNA extraction: Peripheral blood was collected on EDTA pre-coated tubes and then genomic DNA was extracted by a commercial kit (Bioneer, South Korea). The extracted DNA samples were stored at -20°C for further use or immediately subjected to DNA extraction.

Genotyping of CXCL12 (SDF-1): The CXCL12 (SDF-1) gene polymorphism at position +801 SDF-1 3’ A was analyzed by polymerase chain reaction-restriction length polymorphism (PCR-RFLP) method as described previously [15]. Briefly, PCR reaction mixture was made up by addition of the following reagents to a 0.2 ml microcentrifuge tube on ice: 2.5 μl of Taq DNA polymerase buffer (10X), 0.5 μl of Mg Cl2 (stock concentration
1.5 mM), 0.5 μl of each dNTP (dATP, dCTP, dGTP, dTTP) stock concentration of 10 mM), 1 μl of each primer CXCL-12 (SDF-1) 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 cycle of 93°C for 2 min, 93°C for 1 min (denaturation), 1 min at 57°C for annealing of CXCL12 (SDF-1), 72°C for 40 sec (elongation) followed by 30 cycles of 93°C for 20 sec, 55°C for 20 sec and 72°C for 40 sec. During the last 45 sec of first stage 0.3 μl of Taq DNA polymerase was added to the mixture. The amplified PCR product of CXCL-12 (SDF-1) gene covers +801 region with a molecular size of 302 bp. The Sac-1 (Fermentase, Finland) restriction enzyme has merely a restriction site on this region, thus, the fragment will be digested into two 202 and 100 bp fragments following digestion. The digested products were run on a 2.5% agarose gel after adding 4 μl loading buffer (Cinnagen, Iran) and studied on UV transilluminator after staining with ethidium bromide. Studied on Chemi-doc model XRS (Bio.Rad, USA).

**Genotyping of CXCL10 (IP-10):** This gene polymorphism was analyzed by PCR-RFLP method. PCR of this gene was performed as described in CXCL-12 gene amplification except 1 μl rather than 1 μl of prepared DNA and using 57°C annealing temperature. The amplified PCR product of CXCL10 (IP-10) gene covers -1443 regions with a molecular size of 290 bp. The Sac-1 (Fermentase, Finland) has merely a restriction site on this region, thus, the fragment will be digested into two 145 bp fragments following digestion. The digested products were run on a 2.5% agarose gel after adding 4 μl loading buffer (Cinnagen, Iran) and studied on UV transilluminator after staining with ethidium bromide. Studied on Chemi-doc model XRS (Bio.Rad, USA).

**Chemokine assay:** The serum levels of CXCL10 (IP-10) and CXCL-12 (SDF-1) were measured by ELISA (R&D systems, UK) in patients and healthy controls. Assays were performed as manufacturer’s guide lines. The sensitivity of kits was 2 pg/ml and inter and intra-assay assessments of reliability of the kit were conducted.

**Statistical analysis:** Hardy-Weinberg equilibrium was assessed using genotype data. Allele and genotype frequencies were calculated in patients and healthy controls by
direct gene counting. Statistical analysis of the differences between groups was determined by χ² test using EPI 2000 and SPSS software version 13. The p value less than 0.05 were considered significant. The study power was also calculated for each allele and genotype.

RESULTS
Our findings demonstrated that the mean concentration of CXCL₁₂ (SDF-1) in T1D patients and controls were 385.95 ± 44 and 86.1 ± 43 pg/ml, respectively. We also find that the concentration of CXCL₁₀(IP-10) in T1D patients and controls also were 456 ± 56 and 142.6 ± 55 pg/ml, respectively. Results of this study showed that the circulating levels of CXCL₁₀ (IP-10) in T1D patients was approximately 3.5 folds above normal control and the CXCL₁₂ (SDF-1) was 4.5 folds more than controls (Fig 1, 2). At the above figures white and black columns are related to the T1D and Healthy controls, respectively.

![Fig1: CXCL10 (IP-10) circulating level in T1D patients and corresponding control](image1)

Significant difference with control

Fig 1: CXCL10 (IP-10) circulating level in T1D patients and corresponding control

![Fig2: CXCL12 (SDF-1) circulating levels in T1D patients and corresponding control](image2)

Significant difference with control

Fig 2: CXCL12 (SDF-1) circulating levels in T1D patients and corresponding control
Where compared there was a significant correlation between the levels of both chemokines (CXCL_{10} and CXCL_{12}) circulatory levels with the age, and duration of being diabetic in T1D patients and as the diabetes duration and age of patients were increased the chemokine levels were also significantly increased (P< 0.001) (Table 1).

| Table 1: Variation of CXC chemokines levels in diabetes duration and age of patients |
|------------------------------------------|----------------|----------------|----------------|----------------|
| Chemokine             | Duration of T1D | Chemokine       | Duration of T1D | Chemokine       | Duration of T1D |
| CXCL10 (Mean± SEM)    | 1-10 (years)   | 117.6± 16.5     | 243.1± 28.3     | 682.5± 93.7     | 949.5± 25.1     |
| CXCL12 (Mean± SEM)    | 101.9± 15.2    | 209.6± 24.6     | 571.4± 93.8     | 691.7± 16.1     |

A significant difference was observed regarding CXCL_{10} (IP-10) and CXCL_{12} (SDF-1) levels between control, without complication diabetes patients and patients suffering from neuropathy (p<0.003), retinopathy (p< 0.119), neuropathy and retinopathy (p<0.012), nephropathy (p<0.106), retinopathy and ulcers(p< 0.080), nephropathy and neuropathy (P< 0.112). When we compared the circulatory level of CXCL_{12} (SDF-1) according to the gene variants, we observed that the T1D patients with AG genotype had significantly elevated levels of the chemokine 167 (42.3%). T1D patients with GG genotype had minimum 23 (5.8%) CXCL_{12} (SDF-1) level and the mean level of CXCL_{12} (SDF-1) in AA genotype was 15 (3.8%) A statistically significant difference was observed in different genotypes according to the CXCL12 (SDF-1) expression. As shown in table 3 statistical analysis of demographic parameters indicated that, the mean age and gender status of the participants had no markedly
differences which were as following: the mean age of patients was 26.3 years and of control group was 48±12 years. The gender variation of patients was 103 (103%) female and 106 (106%) male and for control group was 101 (101%) female and 88 (88%) male. Our results indicated a significant increased levels of CXCL10 (IP-10) and CXCL12 (SDF-1) in serum of T1D Patients in compare to their related control (Fig-1) and (Fig-2) (p<0.001). Evaluation of the polymorphisms within +801 region of CXCL12 (SDF-1) showed that A/A genotype prevalence was 3.8% in patients and 6.8% in controls. Our results also revealed that the frequency of A/G genotype was 42.3% and 15.2% in patients and controls, respectively. Frequency of the G/G genotype in patients was 5.8% while it was 25.9% in controls (Table 4).

Table 3: Analysis of T1D patients in terms of demographic parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>T1D</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>48 ± 12</td>
<td>30 ± 9.5</td>
<td>p &lt; 0.085</td>
</tr>
<tr>
<td>Gender: M/F</td>
<td>88 / 101</td>
<td>104 / 101</td>
<td>p &lt; 0.9</td>
</tr>
<tr>
<td>Familial history of T1D</td>
<td>-</td>
<td>30</td>
<td>p &lt; 0.003</td>
</tr>
<tr>
<td>FBS (mean ± SE mg/ml)</td>
<td>93.39± 0.79</td>
<td>227.63± 8.95</td>
<td>*p &lt; 0.0001</td>
</tr>
<tr>
<td>HbA1C (mean ± SE)</td>
<td>5.53± 0.13</td>
<td>9.11± 0.23</td>
<td>*p &lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 4: Variation of genotypes and alleles between serum of T1D Patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>T1D n (%)</th>
<th>Control n (%)</th>
<th>OR 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>15 (7.3%)</td>
<td>27 (14.3%)</td>
<td>0.85</td>
<td>-.187-.077</td>
</tr>
<tr>
<td>AG</td>
<td>167 (81.5%)</td>
<td>60 (31.72%)</td>
<td>0.94</td>
<td>-.034-.076</td>
</tr>
<tr>
<td>GG</td>
<td>23 (11.2%)</td>
<td>102 (54%)</td>
<td>0.75</td>
<td>-.005-.009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>T1D n (%)</th>
<th>Control n (%)</th>
<th>OR 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>197 (48.5%)</td>
<td>264 (69.8%)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>213 (51.5%)</td>
<td>114 (30.2%)</td>
<td>2.27</td>
<td>1.7-3.03</td>
</tr>
</tbody>
</table>

Statistical analysis of our data confirmed a significant difference between the two groups (p< 0.001). The frequency of A allele was 197 (25%) and 114 (14.5%) in patients and controls, respectively. 213 (27%) cases of G allele were observed in patients but the frequency of this allele was 264 (33.5%) in controls. Statistical analysis of alleles also exhibited a significant difference between patients and controls (p< 0.0001). Our results showed that all of studied individuals either control or patients showed similar genotype of CXCL10/1443 promoter polymorphism so that all exhibited AG genotype.

DISCUSSION
This case–control study was undertaken to
examine the role of both CXCL\textsubscript{10} (IP-10) / -1443 and CXCL\textsubscript{12} (SDF-1) /SDF-1-3’ A polymorphisms in susceptibility to T1D in Eastern Iranian T1D patients. We have chosen patients and controls from the same ethnic background and all patients and controls shared a similar geographic area for live in Eastern part of Iran. To the best of our knowledge this is the first study to examine the CXCL\textsubscript{10} (IP-10) / -1443 polymorphisms in T1D patients and also the first study which reports the SDF-1 3’ A genetic variant in Iranian and Middle East regional countries (eg: Pakistan, Afghanistan, Turkey, Iraq,...) T1D patients. We have demonstrated here that there was a significant association of the CXCL\textsubscript{12} (SDF-1)/SDF-1-3’ A polymorphism with T1D in Eastern - Iranian. The significance of chemokines and their related receptors, which play a fundamental part in the initiation and development of T1D was investigated over the last decade [3-4]. A transition at position +801 (G to A) SDF-1 3’ A, is described as a common polymorphism in the 3’untranslated region of the CXCL\textsubscript{12}(SDF-1)gene [12]. In our study we showed that 7.3% cases of our patients had A/A genotype, 81.5% emerged with A/G and ultimately 11.2% displayed G/G genotype variant. Although, there is not much studies on the polymorphisms in different diseases, the association of SDF-1 3’Apolymorphisms with T1D, and lung cancer [13-17]. have been reported. More recently we also studied an association between the SDF-1 3’ A polymorphisms with post transfusion occult hepatitis B infection and multiple sclerosis [18]. In agreement with our study in has been demonstrated that SDF-1 3’ A polymorphisms is associated with T1D and also auto-immune thyroid disease [1,13]. Therefore, based on our polymorphisms results, it could be concluded that SDF-1 3’ A heterozygote and homozygote gene types are more susceptible to T1D and the genotypes may play important role in development of T1D. These findings may also apply for differential diagnosis of T1D from the other metabolic disorders. In our study we also demonstrated that circulating CXCL\textsubscript{12} (SDF-1) was increased in T1D patients in compare to control. The overexpression of CXCL\textsubscript{12} (SDF-1) protein in T1D patients in this study probably could be as a result of SDF-1 3’ A polymorphism. Winkler et al., showed that this polymorphism has regulatory effects on CXCL\textsubscript{12}(SDF-1) expression [19]. Additionally,our findings probably can help clinicians to expect approximately the occasion of T1D according to the CXCL\textsubscript{12} (SDF-1) serum levels. It has been shown that
attachment of monocytes to the ICAM-I is facilitated by CXCL_{12} (SDF-1) and induced by TNF-α and IL-1β inflammatory mediators [20]. Furthermore, CXCL_{12} (SDF-1) itself demonstrated to induce CXCL_{8} (IL-8) and CCL_{2} (MCP-1) both of which, pro inflammatory and angiogenic chemokines, thus, CXCL_{12} (SDF-1) may accelerate the development of an inflammatory response in T1D patients [21]. Taken together, our results may explain a mechanism by which the induced levels of CXCL_{12} (SDF-1) in T1D patients may confirm a story that is indirectly regulated by some other inflammatory chemokines. TNF-α and IL-1β showed to be increased in T1D patients. In the other disorders and types cell such as hepatocytes and hepatoma cells we have previously showed increased levels of both CXCL_{12} (SDF-1) and CXCL_{10} (IP-10) in response to TNF-α [11]. Importantly, it is possible that migration and recruitment of CXCR4 expressing leukocytes in response to CXCL_{12} (SDF-1) may lead to development of T1D. As we showed there was a positive correlation between circulatory levels of both studied CXC chemokines CXCL_{10} (IP-10) and CXCL_{12} (SDF-1) and diabetes complications (Table-2). Most of the complications of the T1D, follow a pattern of pro-inflammatory and elevation of these chemokines probably is as result of inflammatory response which occurs during T1D. The CXCL_{10} (IP-10) itself is a pro-inflammatory chemokine that its overexpression is reported elsewhere [11] and CXCL_{12} (SDF-1) also may induce other proinflammatory chemokines (e.g. CCL_{2} (MCP-1) and CXCL_{8} (IL-8)) in T1D. Thus, the elevated levels of these CXC chemokines in T1D patients may probably be related to diabetes complications rather than T1D itself. In other words, the elevated CXC chemokines in T1D could probably be implicated as useful biological tool for prediction of the diabetes associated complications. Hence, according to the enhanced levels of these CXC chemokines (as a paraclinical tool) the occurrence of T1D complications could be expected and this may aid preventing organ failure due to T1D complications.

Therefore, our results in a way, indicated that the serum levels of chemokines could probably be used as a key biomarker in T1D prognosis and it may be concluded that elevated CXCL_{10} (IP-10) and CXCL_{12} (SDF-1) levels assisting the progression of T1D and a relation could be assumed between the levels of the chemokine increased levels of autoimmunity in the patients [22]. In another part of this investigation, we showed
that CXCL\textsubscript{10} (IP-10) was markedly elevated in T1D circulation. In the regulatory region of the CXCL\textsubscript{10} (IP-10) the regulatory motifs for the various signaling transduction pathways including heat shock element, NF-KB, IFN-\gamma regulatory motifs etc... is well documented [11,23]. Thus, up-regulation of this chemokine could be as a result of the presence of these regulatory mediators and related stimuli in T1D patients circulation.

One another possible mechanism to elucidate the increased levels of CXCL\textsubscript{10} (IP-10) could be presence of the other unknown stimulatory and antigenic substances, including plasma protein deposits which may stimulate CXCL\textsubscript{10} production at the levels of mRNA or protein by cellular sources.

**CONCLUSION**

In this study we for the first time evidenced that elevated levels of CXCL\textsubscript{10} (IP-10) is unrelated to its promoter polymorphism (-1443) in T1D patients. Our results regarding CXCL\textsubscript{10} polymorphisms demonstrated that all of T1D diabetes patients and control subjects, displayed same genotype (all Ag). This in a way may confirm that the elevated CXCL\textsubscript{10} levels might be probably related to the above mentioned factors but not to the -1443 single nucleotide polymorphism. Moreover, it could probably be concluded that other environmental and genetic factors (e.g. inflammation, disease state, diabetes complications etc...) rather than this polymorphism are involved in regulation of CXCL\textsubscript{10} expression in T1D. Further, there is not a related study in data base in other ethnic groups to compare and see weather if this polymorphism play a role in other racial and geographical groups. Furthermore, due to the financial limitations and also restrictions in access to some of materials in our country (Iran), unfortunately we could not perform mRNA studies and look at the mRNA levels of these chemokines. Thus, it is very important to address the levels of mRNA changes of these chemokines using Real Time analysis in T1D patients in parallel with protein levels and we hope to do so in our future studies. Moreover, further investigations are necessary to examine the expression of corresponding receptors for these chemokine ligands (e.g CXCR\textsubscript{3}, CXCR\textsubscript{4} and CXCR\textsubscript{7}) on either periphery leukocytes or organ recruited leukocytes (in case of diabetes complications). This help to speculate the possible role for chemokine/receptor axis in T1D. Finally, it is worthy to look at the studied polymorphisms in different racial and geographical groups in Iran (Iranians with Kordish, Lorish, Turkman ... backgrounds) and also neighboring countries such as Iraq,
Afghanistan, Pakistan and Indian racial groups and important by Arabian states located in southern part of the Persian Gulf (UAE, Qatar...).

REFERENCES


