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Increased circulating levels of SDF-1 α (CXCL12) in type 2 diabetic patients is correlated to disease state but is unrelated to polymorphism of the SDF-1 β gene in the Iranian population

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Running title: SDF-1 α level in type 2 diabetes

Abstract

Introduction: Several environmental and genetic factors are believed to influence the onset of diabetes and its complications. It has also been established that cytokines play a key role in the pathogenesis of type 2 diabetes. Previous studies have revealed that polymorphism in the Stromal-Derived Factor 1 α (SDF-1 α) 3'A polymorphism regulates the expression of SDF-1 α . This study was aimed to explore this polymorphism in parallel with SDF-1 β serum level in type 2 diabetic patients.

Material and methods: In this assessment peripheral blood samples were collected from 200 type 2 diabetic patients and 200 healthy controls. DNA was extracted and a PCR-RFLP screening was applied to examine the SDF-1 β 3'A polymorphism. We also applied ELISA to measure serum levels of SDF-1 α (CXCL12).

Results: Our results showed that there were no significant correlations between SDF-1 β 3'A Polymorphism in type 2 diabetic patients when compared to controls. However, our results did show that the serum levels of SDF-1 α (CXCL12) were significantly increased in the patients when compared to controls.

Discussion: Based on the results of this study, we concluded that SDF-1 β 3'A polymorphism do no play a role in the pathogenesis of type 2 diabetes but that elevated serum levels of SDF-1 α (CXCL12) may be important for the eiteology of type 2 diabetes but are unrelated to the SDF-1 α 3'A polymorphism.

Key words: SDF-1 α , Diabetes, Polymorphism

Introduction

Type 2 diabetes is the most frequent type of diabetes but its cause has yet to be clarified [1]. The pathogenesis of type 2 diabetes involves environmental and inherited genetic parameters and it is estimated that this latent disease will affect 200 million people by the year 2010 and 365 million in 2030 [1, 2]. It has been suggested that diabetes has characteristics of an immune disorder as highlighted by the pattern of variation in the profile of cytokine expression seen during the progression of the disease [3,4]. In type 2 diabetic patients, peripheral blood monocytes express more inflammatory cytokines than those from healthy subjects [5]. Cytokines and cytokine-receptor axis have recently been the focus of several studies due to their crucial roles in diabetes and its complications [4] and the impact of cytokine imbalance in type 2 diabetes, with and without nephropathy, has been reported [6]. Increased serum levels of inflammatory cytokines including; IL-18 [7], IL-6 [8] and TNF- α [8], has been documented in type 2 diabetes and its nephropathic complications. Chemokines are a sub-group of cytokines with several biological effects such as recruitment of leukocytes to the sites of injury/infection and inflammation, angiogenesis and angiostasis properties. According to the cysteine in their structure they are classified as CC, CX₃C, C and CXC. Stromal-Derived Factor 1), (SDF-1 also known as CXCL₁₂) fits into the CXC group [9]. SDF-1 is expressed as two protein isoforms, SDF-1 α and SDF-1 β , from a single gene, the differences being in the carboxy terminus

which are changed in SDF-1 β by 4 amino acids encoded on an alternatively spliced exon [20]. The key roles of SDF-1 α (CXCL12) as an inhibitory cytokine of autoimmunity and inflammations raises questions concerning the impacts of this cytokine in the pathogenesis of some diseases including type 2 diabetes.. We were also interested if there was a correlation between a known G801A polymorphism in the SDF-1 β gene (SDF-1 β 3`A) [20] and type 2 diabetes. Therefore, this study was aimed to investigate the serum levels SDF-1 α in parallel with the SDF-1 β 3`A polymorphism in a cohort of Iranian type 2 diabetic patients.

Material and methods

Subject

Peripheral blood samples were collected from 200 type 2 diabetic patients and 200 healthy controls. Patients and controls were selected from within the Rafsanjan population with similar demographic characteristics including sex, age and socio-economical status (Table1). Socio-economical status was measured based on the monthly income. Monthly income lower than €200 was considered as weak, between €200-500 medium and more than €500 high. Lipid levels, proteinuria, estimates of glomerular filtration rate (GFR) and clinical history of the patients and controls are also listed in table 1. The ethical approval of this study was granted by the ethical committee of the Rafsanjan University of Medical Sciences and all patients signed informed consent forms prior to sample

collection.

DNA extraction

Peripheral blood was collected in EDTA pre-coated tubes and genomic DNA was extracted using commercial kits (Bioneer, South Korea). Extracted DNA samples were stored at -20°C for further use .

Polymorphism studies

The G801A polymorphism in the SDF-1 β gene (SDF-1 β 3`A) was analyzed using a polymerase chain reaction-restriction length polymorphism (PCR-RFLP) method as described previously [9]. Briefly, PCR reaction mixtures were made by addition of the following reagents to a 0.2 ml microcentrifuge tube on ice: 2.5 μ l of Taq DNA polymerase buffer (10X), 0.5 μ l of MgCl₂ (stock concentration 1.5 mM), 0.5 μ l of each dNTP (dATP, dCTP, dGTP, dTTP at a stock concentration of 10 mM) and 1 μ l of each primer. The sequence of the forward and reverse primers are: CAGTCAACCTGGGCAAAGCC and AGCTTTGGTCCTGAGAGTCC respectively and were made up to a stock concentration of 25 ng/ μ l. 1 μ l of prepared DNA and sterile double-distilled water to a final volume of 25 μ l was added to all reactions. 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 SDF-1 α primers, 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 μ l of Taq DNA polymerase was added to the mixture. The amplified PCR product of

SDF-1 β gene covers +801 region and had a molecular size of 302bp. There is a unique Sac-1 (Fermentase, Finland) restriction enzyme contained within this region, thus, the fragment containing the G801A polymorphism will be digested into a 202bp and a 100bp sub-fragment following digestion. In the case of the heterozygotic form (A/G) 3 different fragments of 302, 202 and 100bp are visible. In the homozygotic (A/A) form only a 302bp fragment without was observed. The Sac-1HPLC-PCR products were electrophoresed on a 2.5% agarose gel (Cinnagen, Iran) and visualised on a Chemi-Doc model XRS (Bio-Rad, USA) after staining with ethidium bromide.

Chemokine assay

The serum levels of SDF-1 α (CXCL12) were measured by ELISA (R&D systems, UK) in patients and healthy controls immediately after blood collection. Assays were performed according to the manufacturer's guidelines. The sensitivity of kits was 2 pg/ml and inter and intra-assay assessments of reliability of the kit were conducted. Data was only used when the inter- and intra-assays produced scores of CV<14% and CV<0.03%, respectively.

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 χ^2 test using EPI 2000 and SPSS software version 13. A *P* value of less than 0.05 was considered significant. The study power was also calculated for each allele and genotype.

Results:

Statistical analysis of the demographic parameters indicated that, the mean age, gender, and socio-economical status of the participants had no marked differences which were as following; the mean age of patients was 40 ± 6 years and of control group was 40 ± 7 years ($p=0.85$), the gender split of the patients was 124(62%) females and 76 (38%) males and for the control group it was 120 (60%) females and 80 (40%) males ($p=0.9$). In additions, there was no significant difference between groups regarding socio-economical status ($p=0.90$). Our results indicated that proteinuria was significantly increased ($P=0.002$) while estimated GFR was decreased ($P<0.001$) in type 2 diabetic patients when compared to controls (Table 1).

Evaluation of the G108A polymorphisms in SDF-1 β (CXCL12) by *Sac*-I restriction digestion revealed that the prevalence of the A/A genotype was 16(4.0%) in patients and 22 (5.5%) in controls, The frequency of A/G genotype was 90 (22.5%) and 86 (21.5%) in patients and controls, respectively and the frequency of the G/G genotype in patients was 94 (23.5%) and in controls was 92 (23%) (Table 2). Statistical analysis of our data did not show a significant differences between the two groups

($p > 0.766$). The frequency of A allele was 122 (15.3%) and 130 (15.3%) in patients and controls, respectively. 34.8% cases (278) of G allele were observed in patients and the frequency of this allele was 270 (33.8%) in controls (Table 2). Statistical analysis of alleles also did not exhibited a significant difference between patients and controls ($p > 0.893$).

We observed a significant increase in the circulating serum levels of SDF-1 α (CXCL12) in type 2 diabetic patients in comparison to control patients (Fig-1) ($P < 0.001$). The mean concentration of SDF-1 α was 204.24 ± 30.85 pg/ml and 58 ± 10 pg/ml in patients and controls, respectively.

Discussion:

The significant role(s) of cytokine network in the orientation of immune responses is well documented [10]. Several situations including; infection, hormonal conditions and cytokine gene polymorphisms have been described to regulate expression and secretion of cytokines [10]. The main etiological features of type 2 diabetes and its inflammatory complications, such as nephropathy have yet to be identified. But it seems that the immune system plays important roles in etiology and pathogenesis of type 2 diabetes and its associated complications [11]. Therefore, the aim of the current to study was to investigate the potential correlation of the SDF-1 β G801A polymorphism of with type 2 diabetes. In addition, we measured the circulating serum levels of SDF-1 α

in type 2 diabetic patients to determine if its perturbation was associated with type 2 diabetes.

There was no significant differences between type 2 diabetic patients and healthy controls regarding genotypes and alleles of +801 region of the SDF-1 β gene. Therefore, based on the current study it can be concluded that the +801 polymorphisms is not associated with type 2 diabetes in Iranian patients. To the best of our knowledge this is the first study exploring the genetic variation of the SDF-1 β gene in type 2 diabetic patients.

Our second aim was to explore the circulating serum levels of SDF-1 α in patients and controls to determine if there was a correlations between SDF-1 α serum levels and disease state. Our finding showed a very significant increase in circulating levels of SDF-1 α in type 2 diabetic patients when compared to a matched health cohort of patients. Interestingly there are some reports to show increased plasma level of other CXC chemokines such as ENA78, Mig, IP-10, IL-8[12, 13]. In agreement with findings regarding the correlation of urine albuminuria and CXC chemokine SDF-1 α (CXCL12) levels,Wong and colleagues showed elevated levels TNF- α , IL-10, IL-6, IL-18 as well as CXC chemokines such as IL-8, Mig in diabetic patients[13]. We have already shown increased level of SDF-1 α , Gro and IP-10 as inducible chemokins following the stresses of heat

shock [9], under different stimuli and cytokine treatment in primary hepatocytes [14] and hepatoma cell lines[9], thus, the increased level of this chemokine could be a result of osmolarity shock, as we have shown previously [15]. Alternatively, the changes in SDF-1 α could be a response to increased levels of (TNF- α , IFN- γ) or other related inflammatory cytokines [9, 14]

Taking together the results of our current work in combination with previous studies confirm that the aberrant expression of inflammatory cytokines may be the underlying immunological mechanism involved in the development of type 2 diabetes. In agreement with our results a previous study had shown that inhibition of CXCL12 (SDF-1 α) led to decreased glomerulosclerosis, the podocytes number and also albuminuria [16]. Collectively, we can probably conclude from our data and those of Sayyed SG (2009) that SDF-1 α (CXCL12) could be used as a therapeutic target to reduce diabetic complications (e.g. nephropathies). Moreover, our findings suggest that SDF-1 α (CXCL-12) may be involved in the progression of human type 2 diabetes and that SDF-1 α (CXCL12) may also be used as a useful marker to distinguish diabetic complications such as nephropathy from primary renal diseases.

Finally complications of type 2 diabetes are very complex that are associated with several environmental and genetic factors and these aspects of the disease should be examined in further studies in parallel with the current findings.

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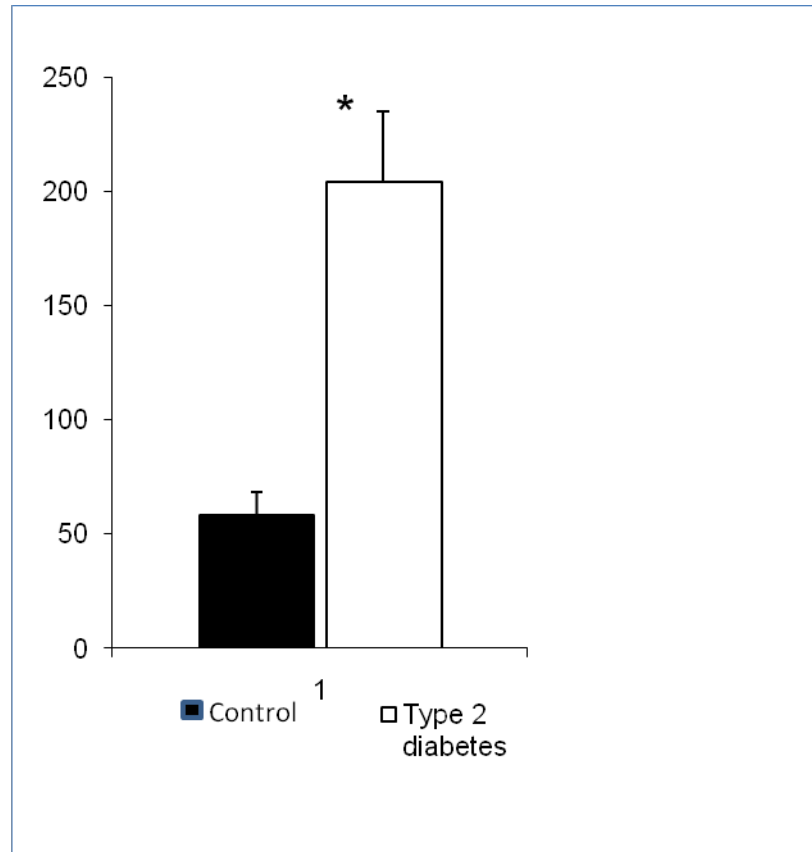


Figure 1. Serum levels of SDF-1 α in type 2 diabetic patients and healthy controls.

* Significant difference in the chemokine serum levels ($P < 0.001$, type 2 diabetic patients with diabetes vs control).

Data are shown as Mean \pm SE.

N	Variant	Healthy control	Type-2 diabetic patients
1	Age (year)	40 ± 7	40 ± 6
2	Sex		
	Female	120 (60%)	124 (62%)
	Male	80 (40%)	76 (38%)
3	Duration of diabetes (years)		10 ± 4
4	Socio-economic status		
	Weak	44 (22%)	48 (24%)
	Medium	94 (47%)	92 (46%)
	High	62 (31%)	60 (30%)
5	Weight (kg)	60 ± 7	50 ± 9
6	Drug therapy	-	insulin
7	Lipid levels		
	Triglyceride (mg/dl)	100 ± 4	350 ± 12
	Cholesterol (mg/dl)	150 ± 6	290 ± 10
	HDL (mg/dl)	40 ± 3	24 ± 2
	LDL (mg/dl)	100 ± 9	180 ± 11
8	<u>Glucose levels (mg/dl)</u>	<u>90 ± 15</u>	<u>230 ± 40</u>
9	Proteinuria (mg/dl)	25 ± 1.5	899 ± 50*
10	Estimated GFR (ml/min)	120 ± 5	72 ± 3 [#]

Table 1. Demographic, socioeconomic conditions and laboratory characteristics of diabetic patient and controls.

* Significant difference in Proteinuria (P< 0.002, t-test, case VS control). Data are shown as Mean ± SE.

[#] Significant difference in estimated GFR (P< 0.001, t-test, case VS control).

Data are shown as Mean ± SE.

<i>Genotype</i>	<i>Patient n (%)</i>	<i>Control n (%)</i>	<i>p value</i>
<i>A/A</i>	16 (4.0%)	22 (5.5%)	> 0.766
<i>A/G</i>	90 (22.5%)	86 (21.5%)	
<i>G/G</i>	94 (23.5%)	92 (23.0%)	
<i>Alleles</i>			
<i>A</i>	122 (15.3%)	130 (16.3%)	> 0.667
<i>G</i>	278 (34.8%)	270 (33.8%)	

Table-2. Frequency of polymorphisms of SDF-1 gene in type 2 diabetic patients and controls.