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Genetic variation of IL-12B (+1188 region) is associated with its decreased circulating levels and susceptibility to type 2 diabetes: a study on south-eastern Iranian diabetic patients

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Running title: IL-12B serum levels and polymorphism in type 2 diabetes

Abstract

Background: Type 2 Diabetes Mellitus (DM) is one of the most common types of endocrine disease and the immune system plays a predominant role in its pathogenesis. The present study was aimed to examine -known gene polymorphisms within IL-12B (+1188) and its circulating serum levels in type 2 diabetic patients from the south-eastern region of Iran and compare them to unrelated controls.

Material and methods: In this descriptive-clinical study, peripheral blood was collected from 114 type 2 diabetic patients and 100 healthy controls. Serum levels of IL-12B were measured by ELISA. Genomic DNA was extracted from peripheral blood samples and polymorphisms at the +1188 position of the IL-12B gene were assessed using PCR-RFLP.

Results: Our findings demonstrated that the AA genotype and the A allele of IL-12B was increased significantly in type 2 diabetic patients when compared to controls. Our results also showed that the circulating levels of IL-12B were significantly decreased in type 2 diabetic patients when compared to control.

Conclusion: According to the findings of the current study, we concluded that IL-12B and its +1188 polymorphism may play a prominent role in the pathogenesis of type 2 diabetes. Further replicative investigations using a larger sample size are essential to identify additional IL-12B genetic variants associated with a risk of type 2 diabetes.

Key words: IL-12B, Polymorphism, Type 2 diabetes

Executive Summary:

- 114 Type 2 diabetes mellitus patients were screened for their circulating levels of IL-12B as well as for mutations within the gene.
- The data shows that in the studied population the circulating levels of IL-12B are lower in patients than in disease-free controls.
- The data also shows that the assessed mutation in IL-12B (+1188) is correlated to a risk of type 2 diabetes but that the evaluated SNP is not the cause of the observed decreased expression of IL-12B in patients.

Executive Summary pointsheadings:

Introduction

1. Type 2 diabetes is a complex disease combining genetic and environmental factors

2. Imbalances in the cytokines that regulate the balance of the immune system are responsible for the pathologies of the disease.

Materials and Methods

43. 114 Type 2 diabetes mellitus patients from the South-East region of Iran were recruited for this assessment.

24. Polymorphisms at position +1188 of the IL-12B gene were assessed by PCR-RFLP.

35. Circulating serum levels of the IL-12B cytokine were measured in the recruited patients by an ELISA assay.

Results

46. The +1188 IL-12B polymorphism correlates to the risk of type 2 diabetes mellitus.

57. The circulating serum levels of the IL-12B cytokine correlates to the risk of diabetes mellitus

68. The polymorphisms at position +1188, situated in the 3'UTR of the IL-12B gene, do not correlate to or influence expression levels of IL-12B in the serum of patients.

Conclusions

9. IL-12B is implicated in the etiology of type 2 diabetes and its decreased expression is correlated to disease state.

10. SNPs at position +1188 of the IL-12B gene are correlated to the disease.

Discussion

11. Although IL-12B is important in the etiology of the disease, mutations at position +1188 do not appear to be the cause of its altered expression levels in the serum of patients, therefore, other factors are responsible for changing the expression of IL-12B during the progression of type 2 diabetes.

Introduction

The type 2 diabetes is the most frequent type of diabetes but the leading causes of this disease have yet to be clarified [1, 2], however, it is believed that the pathogenesis of type 2 diabetes involves, inherited, behavioral and environmental parameters. It is estimated that this latent disorder affected 200 million people in 2010 and will affect 365 million in 2030 [1, 3]. It has

been suggested that diabetes has links with the immune system and is affected by- variations in the profile of cytokine expression [4] and it has been established that cytokines play a critical role in the pathogenesis of the disease [5, 6]. As an example, in type 2 diabetes peripheral blood monocytes secrete more inflammatory cytokines than those from healthy subjects [7].

The expression levels of cytokines vary between individuals and between different societies. The proinflammatory cytokine, IL-12B, was originally purified from a heterodimeric protein consisting -of two disulphide linked subunits of 40 kDa (p40) and 35 kDa (p35), which are encoded by two unrelated genes IL-12B and IL-12A, respectively [8]. Studies showed that the +1188 polymorphism of IL-12B, which is contained in its 3' UTR (untranslated region) is associated with its expression [9]. Accumulating evidence has revealed that levels of inflammatory cytokines including; IL-18 [10], IL-6 [11] and TNF- α [11], are elevated in type 2 diabetes. The association of IL-12B in immunological disorders such as atherosclerosis [12], systemic lupus erythematosus (SLE) [13, 14], nephrotic syndrome [15, 16], graft rejection [17, 18], asthma [13, 19] and type 1 diabetes [20, 21], has been well documented. Previous studies showed that type 2 diabetes is an inflammatory disease [22], but we do not fully understand- which factors are involved its etiology. There are several inflammatory cytokines that can be

implicated in the inflammatory condition of the diseases [23, 24]. IL-12B is an important cytokine for the activation of lymphocytes (acquired immunity) and could be a crucial factor in the development of type 2 diabetes. Therefore, we instigated this study to investigate the relationship between polymorphisms in IL-12B (+1188) in type 2 diabetic patient in the South-Eastern region of Iran. We also examined and compared circulating IL-12B levels in type 2 diabetic patients.

Materials and Methods

Subject

This study population comprised of 114 type 2 diabetic patients (diagnosis based on the [guidelines provided by the](#) American Diabetes Association [25]) recruited from the diabetes clinic of Ali-Ebne Abitaleb hospital, Rafsanjan-Iran and 100 healthy controls that were enrolled from 2008 to 2009. Patients with more than 13 years of diabetes, nephropathy, retinopathy, hypertension or cardio-vascular disease were excluded from the study. Healthy controls were selected from normal healthy adults who were matched with similar medical, sex, age and socio-economical status (Table 1). All samples were collected after obtaining approval from the Rafsanjan University of Medical Sciences ethical committee. We considered the important effects that stress appears to have on the function of immune cells [26] and therefore triaged patient groups by socio-economical status which

was measured based on the monthly income (lower than €200.00: weak, between €200.00-500.00: medium and more than €500.00: high). A written consent form for this research was filled out by both patients and controls prior to sample collection. Fasting blood sugar (FBS) levels and lipid profiles were measured using standard laboratory procedures.

DNA extraction

All peripheral blood samples were collected on EDTA pre-coated tubes. The genomic DNA was extracted by commercial kits (purchased from Bioneer, South Korea). Extracted DNA samples were either directly used for polymorphism analysis or stored at -20°C for further use.

Detection of polymorphisms: IL-12B gene polymorphism at position +1188 were analyzed by PCR-RFLP. PCR reaction mixtures were made up from the 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 1.5 mM MgCl₂, 0.5 µl of each dNTP [dATP, dCTP, dGTP and, dTTP (stock concentration of 10 mM)], 1 µl of each primer (forward: ATTTGGAGGAAAAGTGGAAGA and reverse: AATTCATGTCCTTAGCC), stock concentration of 25ng/ml), 5 µl of prepared DNA and sterile double-distilled water to a final volume of 25 µl. The amplification was performed with the following program of 35 cycles:

one cycle at 94°C for 2min, 93°C for 1 min (denaturation), 53°C for 1 min (annealing), 72°C for 40 sec (elongation). During the last 45 sec of the first stage 0.3 µl of Taq_DNA polymerase was added to the mixture. The expected size of the PCR product was 1046 bp. The amplified PCR product of the IL-12B gene covers the +1188 region and contains a unique *Taq*-1 site which can be used to digest the PCR product -into two fragments of 140 and 906 bp . In the case of the heterozygotic form (A/C) 3 different fragments of 1046, 904 and 140 bp are visible when analysed. In the homozygotic form (A/A) a single 1046 bp fragment -or in the C/C form two products of 906 and 140 bp were observed. The digested products were run on a 2.5% agarose gel after addition of 4 µl of loading buffer (Cinnagen-Iran) and scored on a UV transilluminator after staining with ethidium bromide.

Cytokine level assay

The serum levels of IL-12B were measured by ELISA (R&D systems, UK) in patients and healthy controls immediately after blood collection. Assays were performed as per the manufacturer's guidelines. The sensitivity of kits was 2 pg/ml and inter and intra-assay assessments of reliability of the kit was 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. χ^2 test, t-test and one-way anova analyses were used for calculation of the differences between groups regarding polymorphisms, serum levels of IL-12B and the relationship between polymorphisms versus serum levels of IL-12B, respectively, using EPI 2000 and SPSS software version 14. P values less than 0.05 ~~was~~were considered significant. The study power was also calculated for each allele and genotype.

Results

The mean age in patients and control groups was 40 ± 7 and 40 ± 9 , respectively the age differences between the two groups were not significant (Table 1). Sixty (60%) of control group members were female and 40 (40%) were male while 66 of patients (57.8%) were female and 48 (42.1%) were male. Analysis of socio-economic condition showed that there were no significant differences between patient and control groups (Table 1).

Evaluation of the polymorphism at position +1188 of IL-12B by *Taq* restriction enzyme showed that the wild type IL-12 +1188 A/A genotype [27] frequency was 60 (52.6%) in patients and 36 (36%) in controls. Our results also revealed the frequency of the A/C genotype to be 49 (43%) and 54 (54%) in patients and controls, respectively. The C/C genotype frequency

was 5 (4.4%) and 10 (10%) in patient and control, respectively (Table 2). Statistical analysis of our data confirmed a significant difference between the genotype in two groups ($p < 0.001$). The frequency of *A* allele was 169 (74%) and 126 (63%) in patients and controls, respectively. Fifty nine (25.9%) cases of the *C* allele were observed in patients, whereas, the frequency of this allele was 74 (37%) in controls. Statistical analysis of alleles showed a significant difference between patients and controls ($p < 0.001$) (Table 2). Serum levels of IL-12B in type 2 diabetes patients (1.2 ± 0.294 pg/ml) was significantly decreased compare to the control group (6.69 ± 0.99 pg/ml) ($P = 0.001$) (Figure 1). Our results also demonstrated that serum levels of IL-12B in patients with *A/A*, *A/C* and *C/C* genotypes were 1 ± 0.3 , 0.9 ± 0.2 and 1.3 ± 0.3 , respectively. Statistical analysis revealed that the serum levels of IL-12B were not different among patients with different genotypes.

Discussion

Type 2 diabetes is a complicated and multifactorial chronic disease which commonly appears in adulthood [23]. It appears that immunological factors, including cytokines, are important players in the development and progression of type 2 diabetes [23]. In our present work, we have undertaken a clinical study to examine the role of polymorphisms at position +1188 in

the IL-12B gene as a risk factor for type 2 diabetes in diabetic patients from South-East Iran. Both type 2 patients and healthy control subjects which were recruited in this study belonged to the same ethnic background and all of them shared a common geographical locale in the south-eastern part of Iran. In addition, the subjects were also matched for sex, age, lipid levels, medication, weight, monthly income and levels of education (Table 1). Our results demonstrated a significant decrease in serum levels of IL-12B in type 2 diabetic patients compared to controls. Based on the fact that, cytokines act as a biological network and are regulated by other cytokines within the network, it is possible that reduced serum levels of IL-12 may be related to elevated levels of antagonistic cytokines such as, IL-17A, TGF- β , IL-10 and Th2. IL-17 is an pro-inflammatory cytokine that is produced by Th17 lymphocytes and is involved in the primary immune response against microbes as well as autoimmune diseases [24, 28]. Previous studies showed that IL-17 has an antagonistic effect on the IL-12 production and leads to decreased secretion of IL-12 by the immune cells such as macrophages and dendritic cells [29]. In our previous study we identified increased serum levels of IL-17 as an antagonist of IL-12B expression in type 2 patients [23]. Therefore, we concluded that IL-12B is involved in the pathogenesis of type 2 diabetes. We have also previously shown decreased IL-10 levels in

the type 2 diabetic patients [30]; thus, it can be concluded that, IL-10, as an anti-inflammatory cytokine, has no effect on the reduction of IL-12B expression level in the patients ~~and~~ but it is possible that other anti-inflammatory agents such as TGF- β and Th2 cytokines may affect it.

Wegner et al., reported that IL-12B serum levels of type 2 diabetes patients treated with sulphonylureas (inducing proinsulin-secretion) was increased [31], and Blazhev et al., showed increased serum levels of this cytokine in type-1 diabetic patients [32]. In animal models Carina Malaguti et al., revealed that the serum levels of IL-12B was decreased in non-obese diabetic diacerhein-treated mice [33]. Using a mouse~~iee~~ model of diabetes, Sylvie Trembleau et al., 1995, suggested that the administration of IL-12B as a pro-inflammatory cytokine may be involved in β -cell damage in accordance with differentiation of Th₀ to Th₁ and inflammatory cytokine production [14, 22]. This in turn, facilitates the process of animals developing of diabetes. In addition to IL-12B, investigators also showed that the serum levels of other inflammatory cytokines such as IL-6, IL-18 and TNF- α were also increased in nephropathic type 2 diabetic patients [14, 23]. It seems that, more studies regarding IL-12B production by immune cells of type 2 diabetic patients is-are needed. However, serum levels of IL-12B were decreased with a concurrent increase of IL-17A serum levels in type 2

diabetic patients after aspirin and/or statin therapy (as anti-inflammatory agent) [31] and this could be a possible background cause for down regulation of IL-12B in some patients. The negative correlation between HDL and serum IL-12B levels has been well established, thus, high serum HDL levels can presumably lead to reduction of serum IL-12B levels. ApoA1, as the basic component of HDL, may also prevent dendritic cell maturation and differentiation that in turn leads to reduced IL-12B levels [20]. Overall, the results of the current study on the evaluation of the serum levels of this pro-inflammatory cytokine in type- 2 diabetic patients perhaps suggests that the onset of diabetes is related to immune factors.

In this study we found that IL-12B 3' UTR A-C polymorphism were associated to the pathogenesis of type 2 diabetes but surprisingly, this correlation was found with the increased occurrence of the A/A genotype and the A allele. The A/A is the wild type genotype at the +1188 position of the IL-12B gene [27]. However, in the current study these genotypes and allelic frequencies were increased in the patients when compared to controls. Furthermore, we expected to find increased expression of IL-12B in patients, whereas it was decreased. The reduced expression of IL-12B in the type 2 diabetes may be related to the epigenetic factors (such as micro-RNA, promoter methylation etc.) or the expression of antagonistic factors such as

IL-17, TGF- β and Th2 cytokines that may lead to neutralizing the effect of the wild-type polymorphisms. It is also possible that our population contains an additional polymorphism that enhances IL-12B gene expression. To the best of our knowledge this is the first study to report an association between the IL-12B 3' UTR A-C polymorphism and type 2 diabetes. However Windsor et al., demonstrated a positive correlation between IL-12B gene polymorphisms and the onset of type 1 diabetes [9] and Jian Mei Yang et al., suggested this polymorphism as a risk factor of type 1 diabetes [34]. In particular, based on the result of the present study and others, it can be suggested that polymorphism in the IL-12B gene and altered circulating levels of this cytokine may play a role in the initiation and development of type 2 diabetes. Surprisingly, our current data showed that there were no correlations between serum levels of IL-12B and the polymorphism examined here. A possible explanation may be related to the short half-life of cytokines in the serum. More *in vitro* studies using cultured cells harbouring different polymorphisms in the presence of suitable mitogens may help to resolve this controversy. Our data also showed in our study population the A/A genotype and the increased frequency of the A allele can be considered as possible risk factor for type 2 diabetes. Furthermore, we suggest that for at least some of the complications associated with type 2

diabetes could be related to immune system dysfunction in parallel with other etiological factors such as physiological and pathological conditions involved in this disease.

Type 2 diabetes is a complex disease which appears to be multigenic and further complicated by several environmental factors. Evidence would suggest that cytokines play a key role in the development and/or progression of the disease. Our investigations have revealed surprising results that were unexpected and we need to expand our studies to not only further explore the polymorphisms studied here but to also explore the role of other cytokines in the development of this disease.

Conclusions

In this study 114 type 2 diabetes mellitus patients were recruited from the South-Eastern region of Iran and they were assessed for polymorphisms at position +1188 of the IL-12B. The polymorphism was significantly correlated to the risk of diabetes. Serum levels of IL-12B were also measured in the cohort of patients and were shown to be significantly decreased in patients indicating that expression levels of IL-12B are associated with the risk of diabetes. Surprisingly, our data showed that

serum levels of IL-12B were not correlated to the +1188 polymorphism. We concluded that both the assessed polymorphism and serum levels of IL12B are correlated to the etiology of type 2 diabetes. However, the polymorphism is not linked to IL-12B expression levels and therefore there is at least one, as yet unidentified factor, responsible for regulating expressed levels of IL-12B in our study population.

Future Perspectives:

Diabetes is a multifactorial disease complicated by environmental issues, however, the role of the immune system in the etiology of the disease cannot be ignored. The next few years will focus on SNPs and mutations that are correlated to the disease but what do we do with this data once we understand the chain of events that brings about the onset of diabetes. The solution maybe personalized medicine that accommodates an individual's combination of mutations that caused the disease. With so many factors, such as the cytokines mentioned in this manuscript, currently it is difficult to see a mouse model being designed to cover all the different combinations of gene variants that could be used as a test platform for testing drugs that can realign the immune system and bring the early stages of the disease under control. However, in the future as technologies improve, it may be possible

to prepare a battery of such animals that can be used to test targeted therapies.

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