Gene polymorphism of IL-18 as a pro-inflammatory mediator and susceptibility of diabetes type 1 in Egyptian children.

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ABSTRACT

Type 1 diabetes (T1D) is a chronic immune-mediated disease and is the most common autoimmune disorder in childhood but the disease may become manifest at any age. It is characterized by selective loss of insulin-producing β-cells in the pancreatic islets in genetically susceptible subjects. Interleukin-18 (IL-18) is a proinflammatory cytokine that was originally identified as an interferon-γ inducing factor. It plays a critical role in chronic inflammation, in autoimmune diseases, and in a number of infectious diseases. Functional variants of IL18 gene has been reported as associated with T1D. The purpose of this was to investigate the role of IL-18 gene polymorphism (-607 A/C) in the prediction of T1D in the Egyptian children. One hundred and four patients with T1D and 116 healthy controls, IL-18 promoter genotyping at -607 (rs1946518) position was analyzed with sequence-specific PCR (PCR/SSP). There was no significant association between polymorphisms in SNPs of IL-18 (rs1946518 A/C) and T1D risk in Egyptian population. However, the results suggest an association of genotypes with age at onset of T1D. Our preliminary data suggested that polymorphisms in the IL-18 gene may not contribute to T1D susceptibility. To the best of our knowledge, this study is the first one that deals with IL-18 gene polymorphism in T1D patients in Egypt. Although, further studies with large sample size should be conducted to validate these results in Egyptian population.

Key words: T1D; IL-18; polymorphism; PCR/SSP

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-secreting pancreatic b-cells are destroyed by autoimmune attack. Although the etiology of T1D is not fully understood, it has been suggested to involve interactions between a susceptible genetic background and environmental factors. (Noble and Erlich2012; Lee et al., 2009). Age in type 1 diabetes at symptomatic onset is no longer a restricting factor. (Leslie, 2010). Polydipsia, polyphagia, and polyuria (the classic trio of symptoms associated with disease onset).

T1DM is associated with the appearance of autoantibodies many months or years before symptom onset. These autoantibodies are not thought to be pathogenic but serve as biomarkers of the development of autoimmunity. Characteristic autoantibodies associated with T1DM are those...
that target insulin (Ziegler et al., 1999; Ilonen et al., 2013; Krischer et al., 2015). Individuals with specific HLA genotypes (which encode MHC proteins) — that is, HLADR and HLADQ genotypes (HLADRDQ) — have an increased risk of developing two or more autoantibodies and T1DM (Krischer et al., 2015; Ziegler et al., 2013).

The inflammatory pathway has an important role in the development and complications of T1D (B. Baumann and Salem, 2012). In inflammations events, several interleukins (ILs) could be produced in order to activate specifics immune components and present itself as a potential factor in association to T1D pathogenesis (Hulme et al., 2012; Kunz and Ibrahim, 2009; Reddy, 2004).

Interleukin-18 (IL-18), a member of the IL-1 family, is produced by monocytes, macrophages, and dendritic cells (Srivastava et al., 2010). IL-18 promotes Th1 responses through stimulating interferon (IFN)-γ production by T lymphocytes and natural killer cells in synergy with IL-12 (Nakanishi et al., 2010). Because of the important role of the Th1/Th2 balance in immunological homeostasis, the expression of IL-18 has been correlated with several autoimmune and inflammatory disorders (Shan et al., 2009a).

IL-18 is now recognized as an important regulator of innate and acquired immune responses, including potent stimulation of interferon (IFN)-γ production, enhancement of cell cytotoxicity of natural killer cells, and stimulation of Th cell differentiation (Garlanda et al., 2013; Dinarello et al., 2013; Sedimbiet al., 2013). It plays a critical role in chronic inflammation, in autoimmune diseases, and in a number of infectious diseases (Tanaka et al., 2001; Tucciet al., 2006; Aachouiet al., 2013; Motavaef et al., 2014).

The human IL-18 gene is located on chromosome 11q22.2–q22.3, and the production and activity of IL-18 is regulated by the IL-18 promoter gene (Thompson and Humphries, 2007; Smith and Humphries, 2009; Barker et al., 2011). Accordingly, polymorphisms in the IL-18 promoter gene are attractive candidates due to their impact on pro-inflammatory cytokine production and subsequently on inter-individual disease susceptibility.

The existence of SNPs was investigated at the IL-18 promoter gene (Thompson and Humphries, 2007; Smith and Humphries, 2009; Barker et al., 2011). Recently, these promoter SNPs have been found associated with genetic susceptibility to many autoimmune diseases, such as systemic lupus erythematosus, type 1 diabetes, and rheumatoid arthritis (Dong et al., 2007; Sanchez et al., 2009; Wen et al., 2014). However, divergent results of association have been found in different populations, being necessary replications studies in population with different genetic background.
MATERIALS AND METHODS

Subjects

In the present study, 104 diabetes type 1 patients and 116 healthy individuals as control group were enrolled. (56 males; 48 females). The mean age was 11.08±4.24 with the range of 4-18 years. All patients were examined for the biochemical and diabetic parameters. Demographic and biochemical data of both patients and healthy controls were summarized in Table (1). The diabetes type 1 patients were positive for diabetes. control cases with no history of diabetes in their family. This study was approved by Health and Human Ethical Clearance Committee guidelines for Clinical Researches and following recruitment, the subjects gave informed consent for genetic analysis.

DNA extraction

2 ml of peripheral venous blood was collected from all the patients after an overnight fasting in EDTA tube. Genomic DNA was extracted from whole blood by using the Gentra Puregene Blood Kit (Qiagen Company, Hilden, Germany) according to manufacturer’s instructions. The tubes were centrifuged at 1500 rpm for 10 min. Plasma was separated, aliquoted, and stored at and frozen at -20°C. Extracted DNA was applied to 1% agarose gel to confirm its integrity. The concentration of DNA in all samples was measured by using a Nano drop 2000c (Thermo scientific).

Genotyping of IL-18

Polymorphism at position -607(rs1946518) in the promoter region of IL-18 gene was analyzed by polymerase chain reaction with sequence specific primers (PCR-SSP) method (Zhang P-Aet al 2005). For the position -607 A/C specific PCR, a common reverse primer and 2 sequence specific primers were used. An amplified product of 196 bp was detected. A control forward primer was used to detect an amplified product of 301 bp fragment and acts as an internal positive amplification control (Table 1).

PCR reaction was done in two tubes, one for each allele. Each reaction mixture was consisted of DreamTaq Green Master Mix (2×) (Fermentas, Thermo Fisher Scientific Inc.), by addition of 10 pmol of each allele-specific primer, 10 pmol of reverse primer, and 150 ng of template DNA reaction mixture. In addition, internal positive control primer was added to the reaction mixture. Therefore, for every individual two PCR reactions were performed. All polymerase chain reaction (PCR) were performed in the 2720 thermal cycler (Applied Bio systems). PCR conditions were as follows; denaturation at 94 C for 2 min, followed by seven cycles for 20 s at 94 C, 40 s at 64 C and 40 s at 72 C and 25 cycles for 20 s at 94 C, 40 s at 57 C, 40 s at 72 C and 72 C for 5 min. PCR products were examined by 2% agarose gel electrophoresis stained with ethidium bromide and documented.
Table 1. Primers for IL-18 gene promoter polymorphism.

<table>
<thead>
<tr>
<th>SNP</th>
<th>primer</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward primer</td>
<td>5-GTTGCAGAAAGTGTAAAAATTATTAC-3</td>
<td>196-bp</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5-GTTGCAGAAAGTGTAAAAATTATTTAA-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control forward</td>
<td>5-CTTTGCTATCATTCAGGAA-3</td>
<td>301-bp</td>
</tr>
<tr>
<td></td>
<td>Common reverse</td>
<td>5-TAACCTCATTCAAGACCTCC-3</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 19 (IBM Corporation, USA). Comparisons were made using independent test and results were presented as mean ± SD. Chi-square tests were used to test for differences in allele and genotype distribution between different groups. Odds ratios (OR with 95% CI) were calculated to measure the relative risks in both control and CHB patients. The correlation between variables was determined using Spearman's correlation test. The online tool SNPstats (http://bioinfo.iconcologia.net/SNPstats) was used to perform Haplotype reconstruction from population genotype data, Linkage Disequilibrium parameters (D' and r2) and to identify departure from the Hardy-Weinberg equilibrium. All values were two-tailed, and p < .05 were considered to be statistically significant. P values that remained below .05 after correction for the number of variables (Pc) were considered significant (Bonferroni correction).

RESULTS

Patient's characteristics

In the present study, 104 diabetes type 1 patients and 116 healthy individuals as control group were enrolled. (56 males; 48 females). The mean age was 11.08±4.24 with the range of 4-18 years. All patients were examined for the biochemical and diabetic parameters. Demographic and biochemical data of both patients and healthy controls were summarized in Table (1). The diabetes type 1 patients were positive for diabetes. Levels of HBA1C and BMI showed significant increase in the patients group (P<0.001). Remarkable elevation in HBA1C level was observed in patients compared with controls (r=0.293 and P<0.01). Remarkable elevation in BMI level was observed in patients compared with controls (r=0.679 and P<0.01). Remarkable elevation in age at diagnosis level was observed in patients compared with controls (r=0.293 and P<0.01).
Fig. a. PCR product of IL-18 (-607A/C) for eight controls samples. Lane (1): 100bp DNA ladder. Lane (2, 4, 6, 8, 10, 12, 14 and 16): give A of PCR control cases. Lanes (3, 5, 7, 9, 11, 13 and 15): give C of PCR control cases.

Fig. b. PCR product of IL-18 (-607A/C) for eight patient samples. Lane (1): 100bp DNA ladder. Lane (2, 4, 6, 8, 10, 12, 14 and 16): give A of PCR patient cases. Lanes (3, 5, 7, 9, 11, 13 and 15): give C of PCR patient cases.
Table 2. Demographic and biochemical characteristics of diabetic patients and healthy controls.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group N= 116</th>
<th>Patient group N= 104</th>
<th>P</th>
<th>Correlation with disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>14.96±5.19</td>
<td>11.08±4.24</td>
<td>P&lt;0.01</td>
<td>r= -0.378</td>
</tr>
<tr>
<td>Gender (Male ♂/ Female ♀)</td>
<td>54/62</td>
<td>56/48</td>
<td>NS</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>--</td>
<td>8.16±3.74</td>
<td>P&lt;0.01</td>
<td>r=0.293</td>
</tr>
<tr>
<td>BMI (unit)</td>
<td>10.43±4.99</td>
<td>19.71±1.08</td>
<td>P&lt;0.01</td>
<td>0.679</td>
</tr>
<tr>
<td>HBA1C (unit)</td>
<td>--</td>
<td>9.52±2.42</td>
<td>P&lt;0.01</td>
<td>0.293</td>
</tr>
</tbody>
</table>

All data are presented as mean±SD, body mass index (BMI), not significant (NS), glycosylated hemoglobin (HBA1C)

**Genotyping of IL-18 607A/C SNP (rs1946518)**

IL-18P-607 (A/C) was genotyped by SSP-PCR. The PCR products were measured in comparison to 100bp DNA ladder. The size of PCR product for -607A/C of control cases ([Figure a](#)) and the size of PCR product for -607A/C of patient cases ([Figure b](#)) were 196 bp and control band at 301bp IL-18 p-607 SNPs allele and genotype frequencies of both controls and patients are shown in Table (2). The genotype frequencies of IL-18 (-607A/C) SNP in patients were in HWE (observed data were similar to the predicted data) while controls deviated significantly from the HWE [AA, AC, CC were 1.7%, 57%, 41.3%, respectively (observed)] versus 1%, 60.5%, 38.5 %, respectively non-significant. Table (2) showed that there was significant association between the demographic, biochemical characteristics of diabetes type 1 patients and the difference in their (-607A/C) genotypes. The analysis of haplotype distribution of SNPs in the IL-18 gene promoter region showed no significant association of any haplotype with susceptibility to diabetes (Table 3). To analyze the association of SNPs in IL-18 gene with the patient's clinical presentation (age at onset, HbA1c) the whole patients group was divided in genotype-based subgroups. Patients with the minor allele -607A of IL-18 gene developed diabetes at earlier age at onset than patients with major allele (C) at the same positions, the difference reach significant level (Table 4). However, the minor alleles at each of the two IL-18 loci (-607A) were associated with the disease occurrence at significantly younger age compared to carriers of major alleles (-607C) (Table 4).
Table 3. The genotype and allele frequencies of IL-18 -607 A/C gene polymorphisms in diabetic patients and controls

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Control (N=116) N (%)</th>
<th>Patients (N=104) N (%)</th>
<th>95% Confidence Interval OR (lower-upper)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18 (-607 A/C) genotype (N, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>2 (1.7)</td>
<td>1 (1)</td>
<td>0.553 (0.049-6.194)</td>
<td>NS</td>
</tr>
<tr>
<td>AC</td>
<td>66 (57)</td>
<td>63 (60.5)</td>
<td>1.206 (0.704-2.064)</td>
<td>NS</td>
</tr>
<tr>
<td>CC</td>
<td>48 (41.3)</td>
<td>40 (38.5)</td>
<td>0.885 (0.516-1.521)</td>
<td>NS</td>
</tr>
<tr>
<td>AC/CC vs AA</td>
<td>114 (98.3)</td>
<td>103 (99)</td>
<td>1.807 (0.161-20.224)</td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>60 (30.2)</td>
<td>65 (31.3)</td>
<td>1.170 (0.682-2.008)</td>
<td>NS</td>
</tr>
<tr>
<td>C</td>
<td>162 (69.8)</td>
<td>143 (68.7)</td>
<td>1.807 (0.161-20.224)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4. Demographic Data of diabetic Patients of IL-18 -607 A/C Genotypes.

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>IL-18 -607 A/C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AC</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Age</td>
<td>5.50±0</td>
<td>11.52±4.29</td>
</tr>
<tr>
<td>Age at onset</td>
<td>1.50±0</td>
<td>8.78±3.91</td>
</tr>
<tr>
<td>BMI</td>
<td>14.0±0</td>
<td>19.72±5.08</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present study we demonstrated that IL-18-607 C/A (rs1946518) polymorphisms in Egyptian patients, are not associated with the susceptibility for the development of type 1 diabetes mellitus. In order to, the contradictory results have been obtained by studying the SNPs of IL-18 gene promoter-607C/A in different populations with autoimmune diabetes. To our knowledge, only a few case-control studies reported the significant association of these SNPs with T1DM. In Polish population, the significant association of genotypes with T1DM was found at this loci (Kretowski et al., 2002). In the Chinese (Dong et al., 2007) or Japanese (Ide et al., 2004) populations, significantly different distribution of the -607 genotypes in T1DM patients compared to controls appears due to decreased frequency of patients carrying the AA genotype. In contrast, many studies, including this one, have demonstrated no association of IL-18-137 and -607 polymorphisms with autoimmune diabetes (Martin et al., 2005; Mojtahedi et al., 2006;
Novota et al., 2005; Szeszko et al., 2006). A meta-analysis (6075 cases and 5744 controls) showed that the IL-18 -607 C/A polymorphism may be associated with susceptibility to T1D in Asians, but not in Europeans. 2015 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc.

In our study, we found no association of IL-18-607 genotype, allele or haplotype with T1DM development in Egyptians. However, the results suggest an association of genotypes with age at onset of T1DM.

CONCLUSION

This study observed that there was no significant association between polymorphisms in SNPs of IL-18 genes and diabetes type 1 risk in Egyptian population. Therefore, larger prospective studies are needed to confirm our findings especially in Egyptian populations. In the light of these data, further studies concerning other IL-18 gene polymorphisms will contribute to a better understanding of the pathogenesis of the disease.

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their significance and function.

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