The TGF-β1 Gene Codon 10 Polymorphism Contributes to the Genetic Predisposition to Type 1 Diabetes Mellitus in Egyptian Children

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Abstract

Introduction: Type 1 diabetes mellitus (T1D) results from immune-mediated destruction of the insulin-producing β-cells of the pancreas. Numerous cytokines have been shown to participate in the pathogenesis of T1D. As gene polymorphisms can influence cytokine production or function, they may potentially contribute to genetic predisposition to the disease.

Aim: To investigate the role of TGF-β1 gene codon 10 (+869 T/C) single nucleotide polymorphism (SNP) in genetic susceptibility to T1D in Egyptian children.

Subjects and Methods: The study included 85 subjects, 50 children with T1D (mean age 10.68 ± 3.67 years), and 35 age and sex matched healthy controls. Genotyping of TGF-β1 +869 T/C was done using Real-Time polymerase chain reaction (PCR).

Results: The frequency of the T allele was significantly higher in T1D patients than in control group (71% Vs. 38.6%, p=0.001). While the frequency of the C allele was significantly higher in healthy controls (61.4% Vs. 29%, p=0.001). Homozygous T genotype frequency was significantly increased in patients compared to controls (54% Vs. 20%, p=0.001), while homozygous C genotype frequency was significantly increased in controls (42.86% Vs. 12%, p=0.001). Heterozygous T/C genotype frequency showed no significant difference between the two groups (p=0.76). The TT genotype was associated with an earlier onset and a longer duration of the disease compared to other genotypes (p=0.001). Patients with TC and TT genotypes had significantly higher HBA1c levels than patients with CC genotype (p=0.02).

Conclusion: TGF-β1 gene codon 10 polymorphism is associated with the development of T1D, and T variant is a genetic marker for disease susceptibility in Egyptian children.

Key Words: TGF-β1 – Single nucleotide polymorphism – Type 1 Diabetes Mellitus.

Introduction

TYPE 1 diabetes (T1D) is one of the most common chronic diseases in children and young adults and usually develops as a result of progressive T cell-mediated autoimmune destruction of pancreatic β cells [12]. It is a genetically determined disease and many genes or genetic regions were found to be associated with its induction [3].

To date, several susceptibility loci have been identified on chromosomes 6p21 (HLA class II genes) [4-7], 11p15 (gene for insulin) [8,9], 2q33 (CTLA4) [10], 10p15 (IL2RA) [11], and others to confer genetic risk for T1D [12-16].

Numerous cytokines have been shown to participate in the pathogenesis of T1D [17]. Proinflammatory cytokines are capable of inducing apoptosis of insulin producing cells and mediating the inflammatory process in Langerhans islets, whereas regulatory cytokines likely play a protective role via suppressing the synthesis of proinflammatory cytokines [18].

Polymorphisms in the regulatory and coding regions of cytokine genes have been shown to influence the level of gene expression and in vivo cytokine production, or to alter the structure and function of cytokine molecules. In this way they could contribute to genetic susceptibility and development of the disease [19].

Transforming growth factor-beta (TGF-β) is an immunosuppressive and regulatory cytokine belongs to a family of multifunctional growth factors which have profound regulatory effects on many developmental and physiological processes [20]. Three isoforms are found in humans, TGF-β1, TGF-β2 and TGF-β3, of which TGF-β1 is the most abundant and is highly conserved in primary sequence through evolution [21].

TGF-β1 is produced by numerous cells including Th3 and Treg subsets [22,23]. TGF-β1 could
possibly prevent or slow down the autoimmune mediated destruction of pancreatic Langerhans islets which leads to an absolute lack of insulin production [2,24]. There is mounting evidence that the ability of an individual to produce high or low levels of TGF-β 1 may be genetically predetermined [25,26]. Polymorphism at codon 10 has been reported to be associated with higher TGF-β 1 synthesis [25,27]. Increase or decrease in the production of TGF-β 1 has been linked to numerous diseases [28].

The TGF-β 1 gene is located on chromosome 19q13.1-13.3 [29]. Single nucleotide polymorphism (SNP) has been described, a T/C substitution at position +869 (codon 10) in exon 1 of the gene. This SNP is a good candidate locus because it has been associated with higher circulating TGF-β 1 serum levels resulting from a leucine-to-proline substitution in the signal amino acid sequence of the protein [30,31]. It was speculated that carriage of two copies of the T allele may contribute to susceptibility to T1D via reduced production of the anti-inflammatory TGF-β 1 [32].

In this study, we aimed to determine the association of TGF-β 1 gene codon 10 SNP and the genetic susceptibility to T1D in Egyptian children.

Subjects and Methods

The study was conducted on 85 subjects, the patients group consisted of 50 children with T1D recruited from Health Insurance Endocrine Clinic 6th October Hospital, referred to Pediatric Clinic of National Research Centre. The diagnosis of T1D was established according to the criteria of the American Diabetes Association [1]. All patients required insulin for glycemic control at the time of diagnosis. Conventional regimen involves the administration of two or three injections of insulin, mainly a combination of regular short-acting and intermediate-acting insulin (usually before breakfast and dinner, and at bed-time, respectively) with dose range of 0.5-1.5IU/kg/day, coupled with self-monitoring of blood glucose (SMBG) and adjustment of insulin dosage in response to the individual’s glycaemic control.

Full history taking and thorough clinical examination including anthropometric measurements (height and weight) were done. Body mass index (BMI) was calculated as weight divided by the height squared (Kg/m 2). Relevant laboratory investigations were performed for all patients including fasting and post-prandial blood glucose as well as glycosylated hemoglobin (HbA 1c) levels.

Thirty-five healthy children of same age group and sex distribution with no family history of T1D or any other autoimmune disorders served as a control group.

Informed consents were obtained from the parents of our subjects according to the guidelines of the ethical committee of National Research Centre, Egypt.

Laboratory methods:

Laboratory tests

Venous blood samples were obtained from all subjects on fluoride-containing vacutainer tubes for the fasting and post-prandial blood glucose analysis, and on EDTA-containing vacutainer tubes for the Hb-A1c analysis.

Genotyping

Genomic DNA extraction:

Blood samples were collected using EDTA-containing vacutainer tubes, genomic DNA was extracted from whole blood using the QIAamp DNA extraction kit (Qiagen Hilden, Germany, Cat no. 51304) according to the manufacturer’s protocol.

Genotyping of TGF-β 1 +869 T/C SNP:

For TGF-β 1 +869 T/C genotyping, Real-Time PCR was conducted using TaqMan allelic discrimination method. The oligonucleotide primers for amplification of the polymorphic region of TGF-β 1 were GC088 forward

(5’-CCACCACACCAGCCCTGTTC-3’) and GC088 reverse

(5’-CCGCTTACCAGCTCCATGT-3’). The fluorogenic oligonucleotide TaqMan probes used to detect each of the alleles were: GC088F labeled with 6-FAM to detect the C allele and GC088C labeled with VIC to detect the T allele. PCR amplification was done using 5 µl DNA extract, 1 µl each primer (0.25M), 1 µl each probe (0.3M), 1 µl H2O and 10 µl PCR Master Mix (2x Atlas HotTaq PCR Mix, BioAtlas, Gentaur Biotech., Germany) in a thermal cycler (SLAN Real Time PCR Detection System, LG Lifescience). The thermal cycling profile included an initial step of 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 25s and annealing/extension at 68°C for 1min. To monitor the progress of amplification, the fluorescence was measured at the end of each cycle with excitation/
emission filters of 492/516nm (FAM) and 535/555 nm (VIC). Amplification curves are shown in Figs. (1,2). Subjects, who are positive for either the T allele or the C allele only, are having homozygous genotypes TT or CC respectively. While those, who are positive for both T and C alleles, are of heterozygous genotype (TC).

**Statistical analysis:**

The SPSS package system version 14.0 for windows (Chicago, IL., USA) was used for data management and analysis. Quantitative data was presented as median and mean ± standard deviation (SD). Qualitative data was expressed as frequency (absolute numbers and percent %). Pearson chi-square (χ²) and Fisher’s exact tests were used to compare between independent proportions. Statistical analysis considered to be significant when p-value was ≤0.05. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to estimate the strength of the association. ANOVA test was used for comparison of more than two groups followed by post HOC test if significant.

**Results**

Fifty patients with T1D were included in the study, their age ranged from 4 to 17 years with a median of 11 years. They were 27 females and 23 males with a female/male ratio: 1.17. Median age at disease onset was 7 years, range: 1.5-14.5. Thirty-five healthy children were enrolled as a control group, their age ranged from 3 to 16 years with a median of 10 years, of whom 19 were females and 16 were males with a female/male ratio: 1.18. Descriptive data of the studied T1D patients group is summarized in Table (1).

Analysis of TGF-β 1 gene +869 T/C SNP revealed significant differences in allele and genotype distributions between patients and control groups (Table 2).

The frequency of the T allele was significantly higher in T1D patients group than in control group (71% Vs. 38.6%, p=0.001, OR=3.89, 95% CI=2.03-4.63). While the frequency of the C allele was significantly high in controls compared to T1D patients (61.4% Vs. 29%, p=0.001, OR=0.26, 95% CI=0.13-0.49).

T1D patients' genotype observed frequencies were TT 54%, TC 34% and CC 12%, and in the control group were 20%, 37.14% and 42.86% respectively.

Our results revealed a significantly increased frequency of TT genotype among T1D patients compared to controls (54% Vs. 20%, p=0.001, OR=2.45, 95% CI=1.43-3.40), while CC genotype frequency was significantly increased in controls (42.86% Vs. 12%, p=0.001, OR=0.18, 95% CI=0.05-0.6). Heterozygous T/C genotype frequency showed no significant difference between the two groups (p=0.76, OR=0.87, 95% CI=0.32-2.36).

Patients with TT genotype characterized by an earlier onset of the disease and a longer duration than the TC and CC genotypes (p=0.001 and 0.04, respectively). Glycosylated hemoglobin (HbA1c) was significantly higher in patients with TT and TC genotypes than in those with CC genotype, P=0.02 (Table 3).

**Fig. (1):** Amplification curves of the T allele showing negative cases (flat curves) and positive cases (increased fluorescent intensity above background).

**Fig. (2):** Amplification curves of the C allele showing negative cases (flat curves) and positive cases (increased fluorescent intensity above background).
Table (1): Descriptive data of T1D patients group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>10.68 ± 3.67</td>
</tr>
<tr>
<td>Age at disease onset (years)</td>
<td>7.3 ± 4.03</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>23 (46%)</td>
</tr>
<tr>
<td>Females</td>
<td>27 (54%)</td>
</tr>
<tr>
<td>Disease Duration (years)</td>
<td>3.34 ± 3.35</td>
</tr>
<tr>
<td>Hb A1c (%)</td>
<td>8.39 ± 1.47</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>38.68 ± 15.42</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.39 ± 0.19</td>
</tr>
<tr>
<td>Body mass index</td>
<td>19.21 ± 3.6</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD.

Table (2): Allele and genotype frequencies of TGF-β1 +869 T/C polymorphism in T1D patients and controls.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TID (n = 50)</th>
<th>Controls (n = 35)</th>
<th>P</th>
<th>OR 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>71 (71%)</td>
<td>27 (38.6%)</td>
<td>0.001</td>
<td>3.89</td>
</tr>
<tr>
<td>C</td>
<td>29 (29%)</td>
<td>43 (61.4%)</td>
<td>0.001</td>
<td>0.26</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>27 (54%)</td>
<td>7 (20%)</td>
<td>0.001</td>
<td>2.45</td>
</tr>
<tr>
<td>TC</td>
<td>17 (34%)</td>
<td>13 (37.14%)</td>
<td>0.76</td>
<td>0.87</td>
</tr>
<tr>
<td>CC</td>
<td>6 (12%)</td>
<td>15 (42.86%)</td>
<td>0.001</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. OR odds ratio, CI confidence interval.

Table (3): Some clinical and laboratory parameters in T1D patients according to different genotypes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at disease onset (years)</td>
<td>CC</td>
<td>12.17±1.94</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>8.5±0.16</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>5.46±3.14</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>CC</td>
<td>1.58±0.92</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>2.26±1.4</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>4.41±4.14</td>
</tr>
<tr>
<td>Fasting blood sugar (mg/dl)</td>
<td>CC</td>
<td>178.8±72.93</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>173.81±74.63</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>175.54±87.67</td>
</tr>
<tr>
<td>Postprandial blood sugar (mg/dl)</td>
<td>CC</td>
<td>222.8±136.14</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>263.44±111.18</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>239.42±104.26</td>
</tr>
<tr>
<td>HBA1c (%)</td>
<td>CC</td>
<td>7.18±0.7</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>9.02±1.81</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>8.27±1.17</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD.

Discussion

Type 1 diabetes (T1D) is a multifactorial autoimmune disorder, caused by several environmental and genetic factors [33]. It is one of the most common childhood diseases, requires lifelong treatment, and is associated with increased mortality mainly due to complications that occur later in life [34].

More than three decades of genetic studies have identified several genetic disease variants and a longer list of putative associated genetic loci. These findings have greatly increased our understanding of the genetic background of T1D and have encouraged the development of genetic tools for mapping complex diseases [34,35].

Potential candidates are genes encoding cytokines, molecules that play an important role in the immunological process and inflammation resulting in the destruction of pancreatic β cells [2,36,37]. Several studies have suggested that proinflammatory cytokines and chemokines (IL-1, IL-6, IL-8, TNF-α, and others), Th1 (IFN-γ, LT, IL-2), and Th17 cytokines (IL-17) may be responsible for destructive insulitis [18,38], whereas immunosuppressive and regulatory cytokines Th2 (IL-4), Th3, and Treg cytokines (IL-10 and TGF-β) and cytokine antagonists (e.g. IL-1Ra) likely play a protective role via suppressing the synthesis of Th1 and proinflammatory cytokines [39-41].

In this case-control study we aimed to investigate the role of codon 10 polymorphism within the anti-inflammatory cytokine TGF-β1 gene in the genetic susceptibility to T1D.

TGF-β1 +869T/C genotyping revealed that the frequency of the T allele was significantly higher in T1D patients than in control group (p=0.001), while C allele was significantly higher in healthy controls (p=0.001). Homozygous T genotype frequency was significantly increased in T1D patients (p=0.001), while homozygous C genotype was significantly higher in healthy controls (p=0.001).

Subjects homozygous for the T allele were at significantly increased risk of developing T1D compared with those carrying codon 10 C allele (p=0.001). The C allele was repeatedly associated with increased TGF-β1 production and higher circulating serum levels [25,27,30,31]. As an immunosuppressive and regulatory cytokine produced by numerous cells, TGF-β1 could possibly prevent or slow down the autoimmune mediated destruction of pancreatic Langerhans islets [2,24].
From our results we suggest that TGF-β 1 gene codon 10 T variant is associated with the development T1D. In accordance with Javor, et al. 2010 who studied the possible role of the TGF-β 1 codon 10 SNP in genetic susceptibility to T1D in Slovak population and found that TT homozygotes were significantly more susceptible to develop T1D [32]. In contrast, Jahromi, et al. 2010 found a significant association of TC genotype, but not TT, with T1D in Caucasian patients [42]. However, both studies suggested a role of this SNP in the predisposition to the disease.

Our result showed that TT genotype was associated with an earlier onset of T1D and a longer duration of the disease compared to heterozygous or homozygous C genotypes (p=0.001). The chronic complications of diabetes are related to the duration of the disease. So, when diabetes lasts longer (from 9.5 to 16.5 years), enormous increase of complications was found [43].

We also found that patients with TT and TC genotypes had higher HbA1c levels compared to patients with CC genotype (p=0.02). In the paediatric age group, it has been shown that good glycaemic control is the most crucial factor for prevention of long-term microvascular complications [44]. This can be monitored by HbA1c, which has a strong predictive value for diabetic complications [45].

So, there are many associated risk factors in TT genotype that may contribute to the severity and complications of the disease. Our results suggest that homozygous T genotype is not only associated with the development of T1D, but this genotype may predispose to bad control of the disease and increased risk of future complications. To the best of our knowledge, this study is the first to evaluate association of TGF-β 1 gene codon 10 SNP with the duration of the TID and the glycemic control. So, further studies on a large scale will be needed to give more informative results to clarify these risk factors.

In conclusion, our results suggest that TGF-β 1 codon 10 T variant is associated with the genetic predisposition to T1D and may affect the severity of the disease. Future complex studies on candidate cytokine genes with haplotype analyses are required to fully understand the role of genetic variations and their interactions in genetic predisposition to T1D to improve the prediction, prevention, and therapy of the disease.

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