TNF-α-308 and INF-γ+874 Gene Polymorphisms in Relation to Susceptibility and Severity of Type 2 Diabetes Mellitus among Egyptian Cases

Aafah Elsaid, Mohamed A. Helaly, El-Sayed Z. Hatata, Osama Fouda, Ahmad Settin

ABSTRACT
To explore the association of TNF-α-308 and IFN-γ+874 genetic polymorphisms with type 2 diabetes mellitus (DM) in Egyptian cases. Participants included 207 cases with type 2 DM, 93 males and 114 females. Their mean age was 57 years. They were compared to a control group of 212 healthy unrelated subjects from the same locality. DNA was amplified using PCR with sequence specific primers for detection of polymorphism related to TNF-α-308 (G/A) and IFN-γ+874 (A/T). The combined homozygote pattern of TNF-α-308 (AA) and IFN-γ+874 (A/A) was significantly higher in diabetic cases versus controls (p=0.023) that was manifest (although non-significant) among complicated, uncontrolled cases and cases with marked insulin resistance. Polymorphism related to TNF-α-308 AA and IFN-γ+874 AA may be considered as a genetic biomarker for type 2 DM in Egyptian subjects with potential impact on family counseling and management.

Key words: TNF-α-308, IFN-γ+874, type 2 DM, gene polymorphism, Egypt

INTRODUCTION
The main cause of type 2 diabetes mellitus (DM) is unclear. Recently, it has been recognized as an immune mediated disease in which cytokines play an important role (1,2). Cytokines are key mediators which regulate immune response; and their expression by immune cells depends on several factors such as infection, inflammation, hormonal conditions and also relevant gene polymorphisms (3).

TNF-α-308 is a multifunctional cytokine primarily produced by macrophages and fat cells. It can directly inhibit phosphorylation of insulin receptors’ substrate and reduce glucose uptake by peripheral tissues (4). In human, the TNF-α gene is located within the highly polymorphic major histocompatibility complex region on chromosome 6p21.3 (5). Many studies have shown that single nucleotide polymorphism (SNP) at position -308 G/A was associated with various inflammatory conditions including DM.
Cytokine gene polymorphism in type 2 DM

(6). IFN-γ is a Th1 cytokine which supports the immune system to perform cytolysis of target cells and also was reported to be increased in DM (7). IFN-γ gene intron-1 polymorphisms was speculated to influence immune complex disease susceptibility which is characterized by an imbalance of various immunoregulatory systems (8).

The aim of the present work is to investigate the association of polymorphisms of TNF-α-308 and IFN-γ +874 genes to the susceptibility and severity of type 2 DM among Egyptian cases from the Nile Delta region of Egypt.

MATERIAL AND METHODS

This study was conducted on 207 diabetic patients (93 males and 114 females) with an age range between 40–78 years (57.38±7.67 years). They were selected from the Department of Endocrinology and Diabetes, Specialized Medical Hospital, Mansoura University, Egypt. All patients had a diagnosis of established type 2 diabetes mellitus on the basis of medical history, clinical examination and laboratory tests. Fasting and post-prandial blood glucose were estimated using the glucose oxidase method (spin react kit, Madrid, Spain) (9). Quantitative determination of insulin, C-peptide and glucagon were done by amplified sensitivity immunoassay performed on microtitre plates (INS-EASIA Biosource, Belgium) using monoclonal antibodies directed against distinct epitopes of the corresponding hormone (10). Glycosylated hemoglobin (HbA1c) was measured by quantitative colorimetric method, determined as percent of glycohemoglobin in relation to total hemoglobin (Human GmbH, Germany). Homeostasis model of assessment of insulin resistance (HOMA) was calculated as = Fasting glucose (mg/dl) x Fasting insulin (IU/ml)/405 (11). Exclusion criteria included cases with systemic or blood diseases that may affect the kidney other than DM such as SLE, leukemia and lymphoma and cases with heavy urinary tract infection.

Cases were compared to a control group of 212 unrelated subjects of matched age and sex from the same locality. They were proven healthy and euglycemic by clinical and laboratory tests. A written consent was taken from every participant in this study.

DNA Extraction and amplification

DNA was extracted from anticoagulated whole blood using a special kit (QiAmp DNA blood Qiagen, Hilder, Germany). An amplification refractory mutation system by polymerase chain reaction (ARMS-PCR) was carried out for detection of both polymorphisms. The total volume of reaction mix (25µl) used for TNF-α-308, consisted of 12µl of dream taq green PCR master mix (2X, Fermentas, USA) mixed with 3µl DNA; 3µl of anti-sense primer: 10 pmol/µl 5’-TCTGGGTGTTCTCTCCATCG-3’ with 4µl of either primers of G 10 pmol/µl 5’-ATAGGTGTGAGGCGCATG-3’ or A 5’-ATAGGTGTGAGGCGCATG-3 and 3µl sterile H2O (DNase free) primers in separate tubes. Cycling conditions included an initial denaturation cycle of 95 °C (1 min) followed by 10 cycles each of 95 °C (15 s), 65 °C (50 s), and 72 °C (40 s), followed by 20 cycles each of 95 °C (50 s), 59 °C (50 s), and 72 °C (50 s) finalized with a long extension cycle of 72 °C for 7 minutes. On the other hand, the reaction mix used for detection of IFN-γ +874 gene polymorphism was in the form of a total volume of 20µl containing 1 µl of antisense primer: 100 pmol/µl; 5’-TCA ACA AAG CTG ATA CTC CA-3’, 1 µl of specific A primer :100 pmol/µl, 5’-TTC TTA CAA CAC AAA ATC AAA CAC AAA ATC AAA TCT-3’, 4 µl of dNTPs (2mM), 2.4 µl of MgCl2 (25 mM), 3 µl of buffer (10x), 0.5µl of Taq DNA polymerase (5 U/µl), 5.1 µl of sterile Mili Q H2O and 3µl of genomic DNA. PCR was performed in a thermocycler with the cycles: 95 °C (3min), 10 cycles of 95 °C (15 s), 65 °C (50 s) and 72 °C (40 s), followed by 20 cycles of 95 °C (20 s), 55 °C (50 s) and 72 °C (50 s) and a final cycle of 72 °C (7min). Amplified products were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and visualized on an ultraviolet transilluminator. (12-14).

Statistical analysis

Data were processed and analyzed using the Statistical Package of Social Science (SPSS, version 15). The frequency of studied allelic polymorphisms among cases was compared to that of controls describing number and percent of each and tested for positive association using Fisher’s exact test and odds ratio (OR) with 95% confidence intervals (CI). A minimum level of p < 0.05 is considered significant.

RESULTS

Both TNF-α-308 AA and IFN-γ+874 AA genotypes showed higher frequency among diabetic patients when compared to control subjects, (5.8% vs. 0.9% and 13% vs. 6% respectively) but with no statistical significance (p>0.05) (Table 1).
On the other hand, comparing the combined genotype frequencies among cases and controls, it was noted that cases showed a significantly higher frequencies of the combined genotypes having homozygosity for both rare alleles (AA with AA) or either one of them (AA with AG or AG with AA) (Table 2, p= 0.023). Also, the homozygotic pattern TNF-α-308 (AA) with IFN-γ (AA) was higher in complicated versus non complicated cases, in patients with marked insulin resistance (HOMA >20 (15)) and also higher in uncontrolled patients (HbA1c >7 (16)) but didn’t reach the statistical significance (Table 2).

On the other hand, no statistically significant difference was noted comparing the frequencies of TNF-α-308 (G/A) and IFN-γ +874 (A/T) genotypes and alleles among various case-subgroups regarding: age (<40 vs. >40 years), sex (males vs. females), parental consanguinity (positive vs. negative), family history of DM (positive vs. negative), main blood pressure (hypertensives vs. non-hypertensives) and BMI (overweight/obese vs. normal) (data not shown).

DISCUSSION

Type 2 DM is the most common type of diabetes that is characterized by variable degree of insulin deficiency and resistance. Its prevalence rises markedly with increasing degree of obesity and sedentary life (17). The association of genetic polymorphism of inflammatory cytokines with type 2 DM is largely debatable, but recent findings indicate that certain proinflammatory cytokines are capable of interfering with insulin sensitive glucose uptake and can induce insulin resistance (18).

This study showed that the presence of the rare alleles of TNF-α-308 and IFN-γ +874 genes in combination either in a homozygous of heterozygous forms was significantly higher in Egyptian cases with type 2 DM compared to controls. Also their frequency was higher in complicated cases, in those with marked insulin resistance and with poor controlled glycaemia. These results probably point to the potential impact of the combined association of rare alleles of TNF-α (A) and INF-γ (A) on the susceptibility and severity of type 2 DM.

As an agreement to this study, Kubaszk et al., reported - in their study among Finnish population- that the -308 A allele of the TNF-α gene was associated with two fold higher risk for type 2 DM, and was also a predictor for the conversion from impaired glucose tolerance (IGT) to type 2 DM (19).

Given that approximately 84% of the Egyptian type 2 diabetic cases were carriers of the TNF-α-308 A allele, we can speculate that these cases are high producers of TNF-α as the -308A allele had been shown to increase the transcription and expression of TNF-α and was also found to inhibit insulin signaling and impair insulin secretion (3, 20). Furthermore, the gene frequency of A allele of TNF-α in Egyptian diabetic cases was relatively higher than that reported among other populations like Swedish (21), Northern Irish (22), and

Table 1. Frequencies of TNF-α-308 (G/A) and IFN-γ +874 (A/T) genotype and allelic polymorphisms among type 2 diabetic Egyptian patients compared to control subjects

<table>
<thead>
<tr>
<th></th>
<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Fisher exact (p)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α-308 Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>10 (14.5)</td>
<td>11 (10.4)</td>
<td>0.478</td>
<td>1.46 (0.59-3.66)</td>
</tr>
<tr>
<td>GA</td>
<td>55 (79.7)</td>
<td>94 (88.7)</td>
<td>0.129</td>
<td>0.50 (0.22-1.16)</td>
</tr>
<tr>
<td>AA</td>
<td>4 (5.8)</td>
<td>1 (0.9)</td>
<td>0.080</td>
<td>6.46 (0.71-59.08)</td>
</tr>
<tr>
<td><strong>IFN-γ +874 Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>7 (10.1)</td>
<td>8 (7.5)</td>
<td>0.588</td>
<td>1.38 (0.48-4.0)</td>
</tr>
<tr>
<td>AT</td>
<td>53 (76.8)</td>
<td>92 (86.8)</td>
<td>0.102</td>
<td>0.50 (0.23-1.11)</td>
</tr>
<tr>
<td>AA</td>
<td>9 (13)</td>
<td>6 (5.7)</td>
<td>0.103</td>
<td>2.5 (0.85-7.37)</td>
</tr>
<tr>
<td><strong>TNF-α-308 Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>75 (54.3)</td>
<td>116 (54.7)</td>
<td>1.0</td>
<td>0.99 (0.64-1.52)</td>
</tr>
<tr>
<td>A</td>
<td>63 (45.7)</td>
<td>96 (45.3)</td>
<td>1.0</td>
<td>1.02 (0.66-1.56)</td>
</tr>
<tr>
<td><strong>IFN-γ +874 Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>67 (48.6)</td>
<td>108 (50.9)</td>
<td>0.743</td>
<td>0.91 (0.59-1.40)</td>
</tr>
<tr>
<td>A</td>
<td>71 (51.4)</td>
<td>104 (49.1)</td>
<td>0.743</td>
<td>1.1 (0.72-1.69)</td>
</tr>
</tbody>
</table>
Cytokine gene polymorphism in type 2 DM

Chinese diabetics (23). It is worth mentioning that testing the association of TNF-α -308 with type 1 diabetes among Egyptian cases showed similar results of significantly higher frequency of the AA genotype and A allele in cases compared to controls (24).

Nonetheless, these results are not in agreement with Furuta et al. who did not find any differences in allelic frequencies of the TNF-α -308 (G-A) polymorphism between type 2 diabetes and unrelated controls in Japanese patients(25). This probably points to the potential positive impact of the analysis of combined polymorphic variants of inflammatory genes –as in our cases– rather than of one important single gene polymorphism.

As regard IFN-γ+874 (A/T) gene polymorphism, this study showed that the frequency of AA genotype was higher in Egyptian diabetic cases versus control subjects particularly among uncontrolled cases. These results predict that the Egyptian cases were low producers of IFN-γ as previously reported by Pravica et al.(14). This may draw the attention to the effect of the low production of INF-γ on the development of immune disturbance that gives predisposition to type 2 DM. Similar finding was previously reported by Tsiavou et al., who found increased IFN-γ +874 AA genotype in Greece diabetic populations versus controls (26). In contrast, Arabadadi et al. reported no relation between IFN-γ +874 (A/T) polymorphism and type 2 DM in Iranian diabetic patients(1). This apparent discrepancy may be due to the lack of linking this polymorphism with other important polymorphisms like that TNF gene.

In conclusion, considering the fact that genetic polymorphisms are population specific, it might be speculated that the rare A allele of both TNF-α -308 and INF-γ +874 is associated with type 2 DM predisposing to complications, increased insulin resistance and poor control. These can be considered potential biomarkers with a probable impact on diagnosis and management.

Conflict of interest
Authors declare absolute freedom from any issue pertinent to conflict of interest related to this work.

REFERENCES


