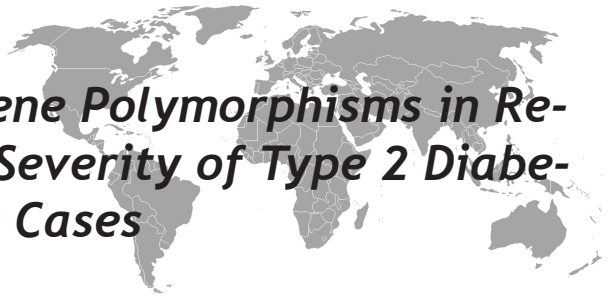

TNF-a-308 and INF-γ+874 Gene Polymorphisms in Relation to Susceptibility and Severity of Type 2 Diabetes Mellitus among Egyptian Cases

Afaf Elsaid[1], Mohamed A. Helaly[2], El-Sayed Z. Hatata[2], Osama Fouda[2], Ahmad Settin[1]

ABSTRACT

To explore the association of TNF-a-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-a-308 (G/A) and IFN-γ+874 (A/T). The combined homozygote pattern of TNF-a-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-a-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-a-308, IFN- γ +874, type 2 DM, gene polymorphism, Egypt

Mısırlı Olgularda TNF-A-308 ve INF-γ+874 Gen Polimorfizmini Tip 2 Diabetus Mellitus Şiddeti ve Duyarlılık İlişkisi

ÖZET

Mısırlı tip 2 diabetus mellitus (DM) olgularında TNF-a-308 ve INF-γ+874 Gen Polimorfizmi ilişkisini araştırmak. Katılımcılar 93 erkek ve 114 kadın tip 2 DM'lu olgudan oluştu. Ortalama yaş 57'ydi. Benzer çevreden farklı 212 sağlıklı kontrol grubu ile karşılaştırıldı. TNF-a-308 (G/A) ve IFN-γ+874 (A/T) ilişkili polimorfizmin tespiti için sekans spesifik primerli PCR kullanılarak DNA çoğaltıldı. TNF-a-308 (AA) ve IFN-γ+874 (A/A)'nın kombine homozigot paterni kontrollerle karşılaştırıldığında diabetiklerde anlamlı yüksekti (p=0.023),bu (anlamlı olmasa da) komplike, kontrolsüz olgular ve belirgin insülin dirençli olgularda belirgindi. TNF-a-308 AA ve IFN-γ 874 AA ile ilgili polimorfizm aile danışmanlığı ve yönetimi üzerindeki potansiyel etkisi ile Mısır bireylerde tip 2 DM için genetik bir belirteç olarak kabul edilebilir.

Anahtar kelimeler: *TNF-a-308, IFN- γ +874, tip 2 DM, gen polimorfizm, Mısır*

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

Mansoura University, [1]Genetics Unit of Children Hospital, [2]Diabetes and Geriatric Unit of Specialized Medical Hospital, Mansoura, Egypt.

Correspondence: *Mohamed A. Helaly, Internal Medicine departement, Specialized Medical Hospital, B.O.Pox 35516, Al-Gomhoria Street, Mansoura, Egypt. E-mail: helaly70@yahoo.com*

Received: 03.01.2012, Accepted: 20.03.2012

(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 (QiAamp 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`-TCTCGGTTTCTTCTCCATCG-3` with 4 µl of either primers of G 10 pmol/µl 5`-ATAGGTTTTGAGGGGCATGG-3` or A 5`-ATAGGTTTTGAGGGGCATGA-3`and 3µl sterile H2O (DNase free) primers in separate tubes. Cycling conditions included an initial denaturation cycle of 95 ºC (1 min) followed by 10 cycles each of 95º C (15 s), 65 ° C (50 s), and 72 ºC (40 s), followed by 20 cycles each of 95 ºC (50 s), 59 ° C (50 s), and 72 ° C (50 s) finalized with a long extension cycle of 72º 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 TCA-3` or 1 µL of specific T primer : 100 pmol/µl, 5`-TTC TTA CAA CAC AAA ATC AAA TCT-3`, 4 µl of dNTPs (2mM), 2.4 µl of Mgcl2 (25 mM), 3µl 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 ºC (15 s), 65 ºC (50 s) and 72 ºC (40 s), followed by 20 cycles of 95 ºC (20 s), 55 ° C (50 s) and 72 ° C (50 s) and a final cycle of 72 ºC (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).

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*

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

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 (hyertensives 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 homozygosus of heterozygous forms was significantly higher in Egyptian cases with type 2 DM compared to controls. Also their frequency was higher in complicated cases, and 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, Kubaszek 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 2**. Distribution of cases in relation to combined genotypes with the rare alleles of TNF-a (A) and IFN-g (A) genes*

	TNF-a / IFN-γ				
Groups	*AA/AA n (%)*	*AA/TA n (%)*	*GA/AA n (%)*	*GA/TA n (%)*	*p value*
Subjects					
Controls (n:87)	*0 (0)*	*0 (0)*	*5 (5.7)*	*82 (94.3)*	*0.02**
Cases (n:53)	*1 (1.9)*	*3 (5.7)*	*7 (13.2)*	*42 (79.2)*	
Complications#					
Negative (n:16)	*0 (0)*	*1 (6.25)*	*1 (6.25)*	*14 (87.5)*	*0.99*
Positive (n:37)	*1 (2.7)*	*2 (5.4)*	*6 (16.2)*	*28 (75.6)*	
HOMA					
<20 (n:35)	*0 (0)*	*2 (5.7)*	*4 (11.4)*	*29 (82.9)*	*0.50*
>20 (n:18)	*1 (5.6)*	*1 (5.6)*	*3 (16.7)*	*13 (72.2)*	
HB1AC					
<7 (n:23)	*0 (0)*	*0 (0)*	*1 (4.3)*	*22 (95.7)*	*0.08*
>7 (n:30)	*1 (3.3)*	*3 (10)*	*6 (20)*	*20 (66.7)*	
BMI					
<30 (n:20)	*1 (5)*	*1 (5)*	*3 (15)*	*15 (75)*	*0.61*
>30 (n:23)	*0 (0)*	*2 (8.7)*	*2 (8.7)*	*19 (82.6)*	

*# Complications included diabetic neuropathy, nephropathy and retinopathy *significant p<0.05*

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

1. *Arababadi MK, Pourfathollah AA, Daneshmandi S, et al. Evaluation of Relation between IL-4 and IFN-γ Polymorphisms and Type 2 Diabetes. Iranian J Basic Med Sci 2009;12(2):100-4.*
2. *Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care 2004;27:813-23.*
3. *Daneshmandi S, Pourfathollah A, Arababadi MK, Hassanshahi G, Rezaeian M, Asiabanha M. Evaluation of relation between IL-4 and IFN- γ polymorphisms and type 2 diabetes. J Mazand Univ Med Sci 2008;18:35-41.*
4. *Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis*

factor-alpha in human obesity and insulin resistance. J Clin Invest 1995;92:2409-15.

5. Wastowski IJ, Peres NT, Simoes RT, et al. Identification of a novel 120 bp allele at the TNFa microsatellite locus. Tissue Antigens 2006;67(4):318-20

6. Hajeer AH, Hutchinson IV. Influence of TNF alpha gene polymorphisms on TNF alpha production and disease. Hum Immunol 2001;62(11):1191-9.

7. Stalenhoef JE, Alisjahbana B, Nelwan EJ, et al. The role of interferon-gamma in the increased tuberculosis risk in type 2 diabetes mellitus. Eur J Clin Microbiol Infect Dis 2008;27:97-103

8. Cantor MJ, Nickerson P, Bernstein CN. The role of cytokine gene polymorphisms in determining disease susceptibility and phenotype in inflammatory bowel disease. Am J Gastroenterol 2005;100:1134-42.

9. Trinder P. Determination of blood glucose using an oxidase peroxidase system with an alternative oxygen acceptor. Ann Clin Biochem 1969;6:24-7.

10. Frier BM, Ashby JP, Nairn IM, Blairs JD. Plasma insulin, C-peptide and glucagons concentration in patients with insulin dependent diabetes treated with chlorpropamide. Diab Metab 1981;7(1):45-9.

11. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis Model of Assessment of insulin resistance and beta-cell function from fasting plasma glucose and insulin concentration in man. Diabetologia 1985;28:412-9.

12. Aldener-Cannava A, Olerup O. HLA-DOB1 "Low-resolution" tying by PCR amplification with sequence-specific primers (PCR-SSP). Eur Immunogenet 1994;21:447-55.

13. Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. ARMS-PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta1 gene polymorphism. Transpl Immunol 1999;7:127-8.

14. Pravica V, Perrey C, Sievens A, Lee JH, Hutchinson IV. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene; absolute correlation with polymorphic CA microsateline marker of high IFN-gamma gene. Hum Immunol 2000;61:863-6.

15. Steven E. Stern, Ken Williams, Eleuterio Ferrannini, Ralph A. DeFronzo, Clifton Bogardus, and Michael P. Stern. Identification of Individuals With Insulin Resistance Using Routine Clinical Measurements. Diabetes 2005;54:333-9.

16. American Diabetes Association. Standards of Medical Care in Diabetes—2011. Diabetes Care 2011;34(suppl.1):S11-61.

17. Sullivan PW, Morrato EH, Ghushchyan V, Wyatt HR, Hill JO. Obesity, inactivity, and the prevalence of diabetes and diabetes-related cardiovascular comorbidities in the US, 2000-2002. Diabetes Care 2005;28(7):1599-603.

18. Shiba T, Higashi N, Nishimura Y. Hyperglycemia due to insulin resistance caused by interferon-gamma. Diabet Med 1998;15:435-6.

19. Kubaszek A, Pihlajamäki J, Komarovski V, et al. Finnish Diabetes Prevention Study. Promoter polymorphisms of the TNF-alpha (G-308A) and IL-6 (C-174G) genes predict the conversion from impaired glucose tolerance to type 2 diabetes: the Finnish Diabetes Prevention Study. Diabetes 2003;52(7):1872-6.

20. Wilson AG, Sympons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997; 94(7):3195-9.

21. Hoffstedt J, Eriksson P, Hellstrom L, Rossner S, Ryden M, Arner P. Excessive fat accumulation is associated with the TNF alpha-308 G/A promoter polymorphism in women but not in men. Diabetologia 2000;43:117-20.

22. Herrmann SM, Ricard S, Nicaud V, et al. Polymorphisms of the tumour necrosis factor-alpha gene, coronary heart disease and obesity. Eur J Clin Invest 1998;28:59-66.

23. Lee SC, Pu YB, Thomas GN, et al. Tumor necrosis factor alpha gene G-308A polymorphism in the metabolic syndrome. Metabolism 2000;49(8):1021-4.

24. Settin A, Ismail A, Abo El-Magd M, El-Baz R, Kazamel A. Gene polymorphisms of TNF-a 308 (G/A), IL-1021082 (G/A), IL-62174 (G/C) and IL-1Ra (VNTR) in Egyptian cases with type 1 diabetes mellitus. Autoimmunity 2009;42(1):50-5

25. Furuta M, Yano Y, Ito K, et al. Relationship of the tumor necrosis factor-alpha -308 A/G promoter polymorphism with insulin sensitivity and abdominal fat distribution in Japanese patients with type 2 diabetes mellitus. Diabetes Res Clin Pract 2002;56(2):141-5.

26. Tsiavou A, Hatziagelaki E, Chaidaroglou A, Koniavitou K, Degiannis D, Raptis SA. Correlation between intracellular interferon-gamma (IFN-gamma) production by CD4+ and CD8+ lymphocytes and IFN-gamma gene polymorphism in patients with type 2 diabetes mellitus and latent autoimmune diabetes of adults (LADA). Cytokine 2005;31(2):135-41.