IS there a relation between TNF-α 308 Promoter Gene Polymorphism and a risk of Coronary Artery Disease In patients with Type 2 Diabetes Mellitus?

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Abstract: The hypothesis that both atherosclerosis and type 2 diabetes mellitus share the common antecedent of chronic inflammatory disorders has been gaining acceptance. In fact, abnormalities in immune system function and inflammatory mediators have been found to be associated with several classic cardiovascular risk factors such as hypertension, dyslipidemia, endothelial dysfunction, clotting activation and insulin resistance. one of the most important factors that promote inflammation and arterial thrombosis, is the proinflammatory cytokine TNF-α. The expression of this cytokine is modulated by a polymorphism located at nucleotide -308 of the TNF-α promoter gene. **Objective**: In this study we investigated the impact of the G-308A TNF-α polymorphism on the development of CAD in type 2 diabetes mellitus patients. **Subjects and Methods**:We studied 40 subjects, who were categorized into 3 groups,15 patients having DM type 2 with CAD as group (I), 15 patients having DM type 2 without CAD as (group II), and 10 apparently healthy volunteers served as control group (group III). all patients and controls were subjected to the followings: complete history taking, clinical evaluation, routine laboratory investigations and detection of TNF α gene (-308) polymorphism by restriction fragment length polymorphism (RFLP). AA and GA genotypes of TNF- α 308 were significantly increase in group I (DM2 with CAD) when compared to group II (DM2 without CAD) and control group, while there was significant decrease in GG genotype in group I when compared to group II and control group. Additionally, there were significant increase of FBG, 2H.P.P blood glucose, T.Cholesterol, LDL and Serum Triglyceride levels in mutant genotypes (AA+GA) when compared to wild genotype (GG), while there was significant decrease of HDL level in mutant genotypes when compared to wild genotype. In conclusion, our study indicates that the TNF-α 308G/A polymorphism may be a potent risk factor for coronary artery disease and associated risk of metabolic diseases in diabetic patients.
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1. Introduction

DM is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both ***(American Diabetes Association,2006).*** Type-2 DM accounts for more than 90% of patients ***(Adeghate et al.,2006***). Type-2 DM is characterized by two major defects, impaired insulin secretion or a decrease in its peripheral action ***(Malecki, 2004).***

Increased cardiovascular morbidity and mortality in patients with Type 2 diabetes is well established; diabetes is associated with twice the risk of incident coronary artery disease (CAD) and ischemic stroke and 2–4 times increased risk of CAD and stroke mortality compared with patients without diabetes ***(Lee et al., 2004)***. Type 2 diabetes leads to early endothelial injury, probably as a result of hyperglycemia, hypertension, diabetic dyslipidaemia and insulin resistance. To this end, endothelial dysfunction is an early marker for the development of both the micro- and macrovascular complications of type 2 diabetes ***(Naruse and King, 2001).***

Endothelial dysfunction is also linked with circulating inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and C-reactive protein (CRP), to the development of both type 2 diabetes and the metabolic syndrome ***(Peter et al., 2002).***

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine of class II HLA-DR locus of the short arm of chromosome 6, which induces cellular responses such as proliferation, production of inflammatory mediators, cell death and serves a variety of functions, many of which are not yet fully understood ***(Jo et al., 2003).*** TNF - α initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. Many studies have further disclosed that this peptide contributes to the development of diabetic complications ***(King and Brownlee, 1996).***

Ample evidence supports a role of TNF- α in the development of cardiovascular disease. TNF- α is expressed in atherosclerotic plaques but not in healthy vessels. In atherosclerotic plaques, TNF- α

may contribute to foam cell formation, to T-lymphocyte activation and to the expression of matrix metalloproteinases that may destabilize the plaque by degrading the extracellular matrix ***(Lee and Libby, 1997).*** Detailed studies also implicated TNF- α in the etiology of insulin resistance, a key feature of type 2 diabetes and a major risk factor for cardiovascular disease ***(Heijmans et al., 2002).***

The TNF-α gene is located in the major histocompatibility complex (MHC) region, and a large number of polymorphisms of its promoter have been described ***(Grohe et al., 2006)***. A single nucleotide polymorphism (SNP) at nucleotide -308 (-308G > A, relative to the transcription start site) in the TNF-α gene promoter region may be important in determining host TNF-α response. The SNP -308G > A has been associated with inducible levels of TNF-α *in vitro*, where the change of a guanidine (common -308G allele) to an adenosine (rare -308A allele) results in differential binding of nuclear factors, leading to six to seven folds increase in the inducible level of TNF-α gene transcription ***(Wilson et al., 1997).*** The -308 polymorphism could potentially affect the cell-type and stimulus specific regulation of TNF-α synthesis at the transcriptional level and the A allele of a common –α 308G/A polymorphism in the promoter region of the *TNF* gene is associated with higher reporter gene activity, ***(Heijmans et al., 2002).***

The aim of this study was to investigate the impact of the G-308A TNF-α polymorphism on the development of CAD in type 2 diabetes mellitus patients.

2.Subjects and methods

Subjects

We studied 40 Egyptian subjects, who consecutively attended out patient clinic of the Internal Medicine Department of Tanta University. These subjects were categorized into 3 groups,15 patients having type 2 DM with CAD as group (I), 15 patients having type 2 DM without CAD as (group II) with no history of CAD, and no signs of ischemic changes on electrocardiogram and no ischemic changes during sub-maximal

stress testing ***(Bacci et al., 2002)***, and 10 apparently healthy volunteers served as control group (group III). Patients were classified as having DM2 according to the American Diabetes Association criteria for the diagnosis and classification of diabetes ***(Hotamisligil et al.,1993)***. The diagnosis of CAD was based on World Health Organization (WHO) criteria ***(Alpert et al., 2000***). After informed consent was obtained from the patients and control subjects, we conducted a detailed interview and all patients and controls were subjected to the followings: complete history taking, clinical evaluation as [blood pressure measurements, and body mass index (BMI) calculation (the ratio of weight in kilograms divided by the square of height in meters)], routine laboratory investigations as (fasting, 2 hours postprandial blood glucose levels, total serum cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides serum levels which were determined by standard biochemical methods) and specific laboratory investigation included detection of TNF α gene (-308) polymorphism by restriction fragment length polymorphism (RFLP).

Methods

Venous blood samples were collected in the morning after an overnight fast about 12 Hr. and 2h after oral ingestion of about 75 gm glucose. The fasting blood samples were divided as follows: 2.0 ml were put into an EDTA vacutainer tubes used for genomic DNA extraction. The remainder fasting blood samples and also post prandial samples were put into clean tubes and allowed to clot in water bath at 37C, then centrifuged and the serum obtained were collected into clean dry tubes for other routine investigations.

Extraction of genomic DNA from whole blood:

By salting out technique based on the method of ***(Miller et al., 1988),*** by using Masterpure TM genomic DNA purification kit for blood use provided by *Epicentre Technologies, Madison, (U.S.A.)*.

DNA amplification by Polymerase Chain Reaction (PCR):

DNA of each sample was amplified by the polymerase chain reaction (PCR) amplification kit *(Stratagene)* of DNA fragments using specific oligonucleotide primers *(Sigma)*: sense (5'-AGG CAA TAG GTT TTG AGG GCC AT-3'), antisense (5'-TCC TCC CTG CTC CGA TTC CG-3'). The cycling conditions were: two cycles at 94°C for 3 min., 60°C for 1min., 72°C for 1 min., 35 cycles at 94°C for 1 min.,60°C for 1 min., 72°C for 1 min.; two cycles at 94°C for 1 min., 60°C for 1 min. and 72°C for 5 min.

TNF-α (-308) genotype was determined by Restriction fragment length polymorphism (RFLP) method:

The PCR product of 107 bp was digested with the *NcoI* restriction enzyme *(Fermentas, St. Leon-Rot, Austria)* into two fragments of 87 and 20 bp, respectively. The products of 87 and 20 bp represented the G allele, whereas undigested PCR products represented the A allele. The products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining and visualized by UV light. TNF- α (-308) alleles were classified as G (wild type) or A(mutant) ***(Fig.2).***

Statistical analysis:

Analysis of the data was performed by using the computer program SPSS version 16. Results were expressed as mean ± SD, and differences between the means of different variables were tested using the student t-test. Significance was accepted at the level of $P<0.05$.

3.Results

The characteristics of the 30 diabetic patients and 10 healthy controls are shown in ***Table 1***, DM type 2 patients were divided into two groups, group 1 with coronary artery disease (CAD) while group 2 without CAD compared to control group (group3). All groups were matched as regard age and sex ($P>0.05$) while there was significant difference between the three groups as regard BMI ($p<0.05$), systolic and diastolic BP ($p<0.05$).

Table (1): Comparison between the studied groups as regarding their clinical data

	Group I N=15	Group II N=15	Group III N=10	Tests of significance F-test	*p*-value	Turkey's test
Age/y						
Range	41-69	40-66	37-70	0.09	0.91	>0.05
Mean ± SD	53.3±8.9	52.6±9.3	54.3±10.8			
Sex	**No %**	**No %**	**No %**	χ^2		
Male	9 60.0	11 73.3	4 (40.0)	2.8	0.25	>0.05
female	6 40.0	4 26.7	6 (60.0)			
BMI						P1: <0.05
Range	25-36	24-31	22-28	11.34	<0.05	P2: <0. 05
Mean ± SD	29.9±3.4	27.3±1.9	24.9±1.9			P3: <0.05
Systolic BP (mmHg)						P1: <0.05
Range	130-180	110-160	110-145	10.74	<0.05	P2: <0.05
Mean ± SD	147.0±16.9	132.3±16.2	118.0±11.4			P3: <0.05
Diastolic BP (mmHg)						P1: <0.05
Range	85-110	80-105	70-90	12.19	<0.05	P2: <0. 05
Mean ± SD	98.0±9.4	88.7±10.9	78.6±7.8			P3: <0.05

*P*1: comparison between group I & II
P2: comparison between group I & III
*P*3: comparison between group II & III

Table 2 shows increase in TNF- α AA and GA genotypes in group I (DM2 with CAD) when compared to group II (DM2 without CAD) and control group ($p<0.05$), while there was significant decrease in GG genotype in group I when compared to group II and control group ($p<0.05$). Also, there were significant increase of A allele (mutant type) and decrease of G allele (wild type) in group I when compared to group II and group III ($p<0.05$).

Table (2): Comparison between studied groups as regarding their TNF-α genotypes

	Group I N=15	Group II N=15	Group III N=10	Tests of significance	*p*-value
Genotypes	**No %**	**No %**	**No %**	χ^2	
AA	4 26.7	2 13.3	0 0.0	9.9	0.04
GA	7 46.7	5 33.3	1 10.0		
GG	4 26.7	8 53.3	9 90.0		
G allele	15 50.0	21 70	19 95	11.4	0.03
A allele	15 50.0	9 30	1 5		

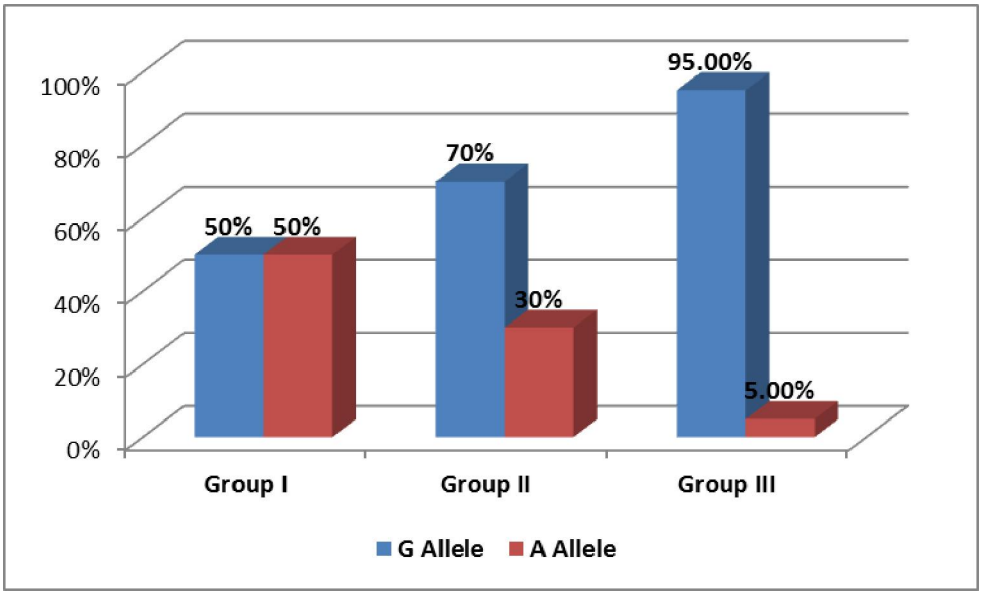

Fig (1): Comparison between studied groups as regarding their allelic frequencies of TNF-α shows significant increase of A allele (mutant type) and decrease of G allele (wild type) in group I when compared to group (II) and group III ($p<0.05$).

Table 3 shows significant increase of FBG and 2H.P.P levels in mutant genotypes (AA+GA) when compared to wild genotype (GG) (*p* -value<0.05). For lipid profiles, there was significant increase of T.Cholesterol, LDL and S.TG in mutant genotypes when compared to wild genotype (*p* <0.05), while there was significant decrease of HDL level in mutant genotypes when compared to wild genotype (*p* -value<0.05).

Table (3): Laboratory parameters distribution according to TNF α – 308 genotypes in diabetic patients with and without CAD

	GG genotype N=12	**AA+GA genotype N=18**	**Tests of significance t-test**	***p*-value**
FBG mg/dl				
Range	125.2-181.1	117.0-205.3	3.6	0.01
Mean ± SD	145.2±15.0	168.6±19.2		
2 H.P.P mg/dl				
Range	195.3-325.6	165-384.4	3.3	0.02
Mean ± SD	247.5±42.9	305.1±48.6		
T.Cholesterol mg/dl				
Range	200.2-275.5	211.4-290.5	3.1	0.04
Mean ± SD	237.6±20.3	260.1±18.4		
LDL mg/dl				
Range	110.1-167.2	115.7-181.6	3.1	0.04
Mean ± SD	133.8±13.7	163.2±18.0		
HDL mg/dl				
Range	42.8-51.0	35.0-46.1	3.6	0.01
Mean ± SD	46.0±2.6	38.3±3.2		
S.TG mg/dl				
Range	105.2-175.7	138.3-351.2	3.6	0.01
Mean ± SD	143.8±30.0	199.6±47.7		

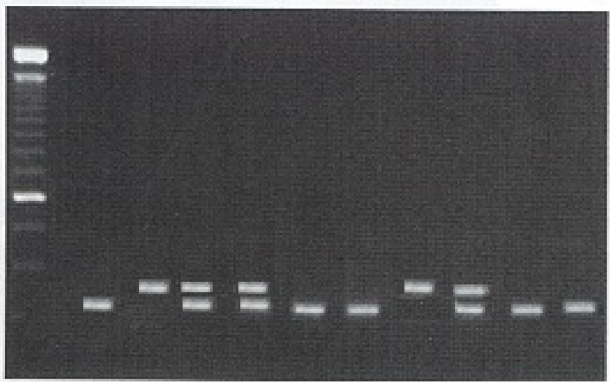

Fig (2): 2% agarose gel electrophoresis for 87-bp and 107-bp PCR products represent the three band patterns of the genotypes. Lane 2,6,7,10,11 represent (GG) genotype, Lane 4,5,9 represent (GA) genotype while Lane 3,8 represent (AA) genotype. Lane 1 is DNA ladder.

4.Discussion

The hypothesis that both atherosclerosis and type 2 diabetes mellitus share the common antecedent of chronic inflammatory disorders has been gaining acceptance. In fact, abnormalities in immune system function and inflammatory mediators have been found to be associated with several classic cardiovascular risk factors such as hypertension, dyslipidemia, endothelial dysfunction, clotting activation and insulin resistance (***Pausova et al., 2000***).

Growing evidence shows that inflammation plays a central role in the pathogenesis of acute myocardial infarction (AMI) (***Elkind, 2006***). Among the factors that promote inflammation and arterial thrombosis, one of the most important is the proinflammatory cytokine TNF-α ***(Chen et al., 2008).*** The expression of this cytokine is modulated by a polymorphism located at nucleotide -308 of the TNF-α promoter gene ***(Abraham LJ and Kroeger KM, 1999)***.

The reported association of G-308 A polymorphism of the TNF-α gene with myocardial infarction and coronary artery disease (CAD) has generated continuing interest. Considering both the potential effects of the 308 A allele on TNF-α and the putative association between TNF-α and cardiovascular disease, a logical hypothesis would be that the 308 A allele is associated with increased risk of cardiovascular disease ***(Ghazouani et al., 2009).***

In this study we investigated the association of -308 G/A polymorphism of TNF-α gene with CAD in type 2 diabetes mellitus**.**

The present study showed that the mean values of BMI were significantly increase in all diabetic patients when compared to the control group, also there were significant increase of BMI in diabetic patients with CAD when compared to diabetic patients without CAD. This is in agreement with ***Vendrell et al. (2000***) who demonstrated significant higher values of body mass index (BMI) in diabetic patients with CAD.

As regarding BP, the present study showed significant increase of both systolic and diastolic BP in all diabetic patients when compared to the control group, also there were significant increase in diabetic patients with CAD when compared to diabetic patients without CAD. This is in agreement with ***Ann (2012)*** who suggested that diabetes increases the risk of developing high blood pressure and other cardiovascular problems, because diabetes adversely affects the arteries, predisposing them to atherosclerosis (hardening of the arteries). Atherosclerosis can cause high blood pressure, which if not treated, can lead to blood vessel damage, stroke, heart failure and heart attack.

As regarding TNF- α genotypes, this study showed significant increase in the number of diabetic patients with AA and GA genotypes and decrease in the number of GG genotype in group I when compared to group II and control group, also there was significant increase of A allele (mutant type) and decrease of G allele in group I when compared to group II and control group. This is in agreement with ***Vendrell J et al. (2003***) who revealed that the CAD patients with type 2 DM displayed a greater prevalence of -308 TNF-α A allele than controls or diabetic patients without MI.

On the other hand, ***Heijmans et al., 2002*** suggested that the - 308G/A polymorphism in the promoter of the gene encoding *TNF* strongly contributed to the risk of diabetes in a population-based cohort of elderly subjects aged 85 years and over and homozygosity for A-allele conferred a more than four-fold increased risk of diabetes. Additionally, ***Keso et al. (2001)*** and ***Antonicelli et***

al., (2005) suggested that the percentage of −308 TNF-α AA homozygous subjects was higher among the population with CAD and high plasma levels of biochemical ischemic markers have been found among AA+AG TNF-α-308 genotype carrier individuals, which showed that they would likely be affected by more severe ischemic damage than the rest of the population. Furthermore, the homozygous A allele carriage of this polymorphism has been associated with unstable angina and susceptibility to plaque formation ***(Bernard et al., 2003).***

Controversially, ***Reschner et al. (2008)*** suggested that there is no association between the TNF-α gene G (–308) A polymorphism and MI in *Slovene* patients with DM2. They added that, there was significantly higher serum TNF-α level in DM2 patients with the GG genotype than with AG genotype. They explained that, this discordance may be due to the multi-factorial nature of the MI, to differences in study design or to genetic heterogeneity within and between the populations studied.

As regarding laboratory parameters distribution according to TNF Alpha -308 genotypes, there was significant increase of FBG and 2H.P.P levels in mutant (GA+AA) genotypes when compared to wild (GG) genotype. ***Cave et al. (2008)*** suggested that TNF-α-308 G/A polymorphism deteriorates insulin signaling and reduces glucose uptake, mediating the relationship of insulin resistance with manifestations of the metabolic syndrome. ***Elva et al. (2011***) reported that the TNF-α − 308G/A genotype was strongly associated with DM2 family history, and they considered this genotype as a risk factor for DM2 in the population. Recently, ***Curti et al., (2011)*** and ***Maira et al. (2012)*** assessed the association of the TNF-α -308 G/A polymorphisms with the risk for obesity and metabolic diseases.

Also as regarding lipid profiles, there was significant increase of total cholesterol, LDL, and S.TG levels in mutant (GA+AA) genotypes when compared to wild (GG) genotype, while there was significant decrease of HDL in mutant genotypes when compared to the wild genotype. ***De Fabiani et al. (2001***) reported that TNF-α raises plasma TG by increasing the concentration of free fatty acids (FFA), the substrate for TG synthesis, and by diminishing the clearance of TG-rich lipoproteins (VLDL) from circulation. TNF-α increases the FFA production from both adipose tissue and liver. In human adipose tissues, TNF-α may increase hepatic cholesterol synthesis by stimulating the HMG-CoA reductase activity, the rate-limiting enzyme in the cholesterol biosynthetic pathway TNF-α may decrease hepatic cholesterol catabolism and excretion.

In conclusion, our study indicates that the TNF-α 308G/A polymorphism may be a potent risk factor for coronary artery disease and associated risk of metabolic diseases in diabetic patients. Further studies on longer scale of subjects are recommended to confirm this link and to use TNF Alpha level as a marker for early diagnosis of CAD development in type 2 diabetes mellitus.

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