

Association of the C242T polymorphism of the p22 phox gene with advanced carotid atherosclerosis in type 2 diabetes

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Received May 5, 2008; Accepted June 25, 2008

DOI: 10.3892/mmr_00000012

Abstract. Reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase is an important source of superoxide (O_2^-) in human blood vessels. A critical component of this oxidase is the p22phox protein, which is encoded by the cytochrome b-245 α (CYBA) gene. Studies have suggested a possible association between polymorphisms of the CYBA gene and susceptibility to atherosclerosis in diabetes mellitus (DM). To address this hypothesis, we examined the relationship between the C242T polymorphism of the p22 phox gene and carotid intima/media thickness (IMT) as a factor of atherosclerosis in a group of type 2 DM patients. The study included 40 type 2 diabetic patients and 20 healthy individuals as controls. All patients and controls were matched in terms of age, gender, lipid profile, blood pressure and body mass index (BMI). The C242T p22 phox polymorphism was investigated by RFLP-PCR, and carotid IMT was measured by color duplex scanning. Diabetic subjects with the T242C and T242T genotypes displayed a significantly lower average of carotid IMT (mean 1.033 ± 0.143 mm, range 0.85-1.25, $P < 0.0001$) than did those with the C242C genotype (mean 1.36 ± 0.177 mm, range 0.98-1.63) despite no differences in other risk factors. The average carotid IMT of both diabetic groups was significantly higher than that of the controls (mean 0.828 ± 0.072 mm, range 0.72-0.86, $P < 0.0001$). In non-diabetic subjects, the average carotid IMT of the TC+TT group did not differ from that of the CC group (0.81 ± 0.08 vs. 0.84 ± 0.069 mm, $P > 0.05$). There was a significant positive correlation between carotid IMT and

blood glucose level and duration. Our findings demonstrate that the CC genotype of the p22 phox gene is a risk factor for the progression of atherosclerosis in diabetic patients.

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The worldwide prevalence of DM has risen dramatically over the past two decades. In 2000, DM affected an estimated 171 million individuals worldwide, and its incidence is expected to rise to 300 million by the year 2025 and to 371 million by 2030 (1). The incidence of both type 1 and type 2 DM varies considerably according to geography; it is higher in developing than in developed countries. The prevalence of DM also varies among different ethnic populations within a given country. This variability is likely due to genetic and environmental factors. Type 2 DM is the predominant form of diabetes worldwide, accounting for approximately 90-95% of cases globally. The prevalence of type 2 DM is expected to rise more rapidly in the future due to increasing obesity and reduced activity levels (2).

Prolonged exposure to hyperglycemia is now recognized as the primary causal factor in the pathogenesis of diabetic complications (3). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and the failure of various organs, in particular the eyes, kidneys, nerves, heart and blood vessels (4). Hyperglycemia induces a large number of alterations in vascular tissue that potentially promote accelerated atherosclerosis. This effect is mediated by three major mechanisms: i) The modification of macromolecules which occurs, for example, by the formation of advanced glycation end products (AGE). These AGE-modified proteins can augment the production of pro-inflammatory cytokines and other inflammatory pathways in vascular endothelial cells. ii) The promotion by the diabetic state of oxidative stress mediated by reactive oxygen species (ROS) and the carbonyl group. iii) The activation of protein kinase C (PKC). Importantly, these mechanisms do not work independently. For example, hyperglycemia-induced oxidative stress promotes the formation of AGE and PKC activation (5,6).

Oxidative stress is widely invoked as being a pathogenic mechanism of atherosclerosis. Under normal circumstances, free radicals are rapidly eliminated by antioxidants such as glutathione, vitamin C and vitamin E. Disturbance in the balance between the production of ROS (free radicals) and

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Abbreviations: AGE, advanced glycation end products; CYBA, cytochrome b-245 α gene; IMT, intima/media thickness; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; O_2^- , superoxide; PKC, protein kinase C; RFLP-PCR, restriction fragment length polymorphism-polymerase chain reaction; ROS, reactive oxygen species

Key words: C242T polymorphism, p22 phox gene, carotid atherosclerosis, type 2 diabetes

antioxidant defenses leads to oxidative stress and, consequently, to tissue injury (7). Hyperglycemia can increase oxidative stress through several pathways. A major mechanism appears to be the hyperglycemia-induced increased production of intracellular ROS. There is also evidence that hyperglycemia may compromise natural antioxidant defenses. Reduced glutathione content, as well as reduced vitamin E, has been reported in diabetic patients. Moreover, plasma and tissue levels of vitamin C are 40-50% lower in diabetic patients than in non-diabetics (4,8).

A number of known free radicals occur in the body, including superoxide (O_2^-), hydroxyl radicals and hydrogen peroxide. Multiple factors, both exogenous or endogenous, contribute to the production of O_2^- and other free radicals. These include exposure to radiation, the smoking of tobacco and ischemic reperfusion injury. In addition, a variety of enzyme systems are capable of generating significant amounts of free radicals during their catalytic cycling, such as reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H]-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases and nitric oxide synthase. NAD(P)H oxidases appear to be the principal source of O_2^- production in several vascular diseases, including DM, hypertension and atherosclerosis (9).

NAD(P)H oxidase is a membrane-associated enzyme that catalyzes electron transfer to oxygen using NAD(P)H as an electron donor to produce O_2^- . It was originally discovered in neutrophils, where it is a potent source of large amounts of O_2^- during phagocytosis and plays a vital role in non-specific host defense. Structurally, NAD(P)H oxidase is a multi-subunit enzyme complex comprising p47phox, p67phox, gp91phox, p22phox and Rac-2 proteins. The p22phox subunit is required for oxidase activity in smooth muscle cells, and is expressed in human coronary arteries (10).

The p22phox protein is encoded by the cytochrome b-245 α (CYBA) gene, located on chromosome 16q24. Several allelic polymorphisms in the p22 phox gene have been reported to have functional consequences in humans. An important one is the C242T polymorphism, which results in the replacement of histidine by tyrosine at amino acid position 72, a potential hem binding site. The C242T polymorphism has a strong influence on the modulation of p22phox expression, NAD(P)H oxidase activity and vascular O_2^- generation. This leads to significant functional variation in vascular oxidative stress between individuals, and therefore to variation in their susceptibility to atherosclerosis (11).

Accordingly, we sought to examine the presence of the C242T polymorphism in a group of Egyptian type 2 diabetic patients, and to investigate a possible relationship between this polymorphism and the development of carotid atherosclerosis as measured by carotid duplex scanning.

Patients and methods

Patients and samples. The study population included 60 subjects, 40 patients diagnosed with type 2 DM and 20 age- and sex-matched non-diabetic apparently healthy controls. The patients comprised 24 males and 16 females with an age range between 45 and 60 years (mean 52.1 ± 4.95) selected from the Internal Medicine Department of Tanta University Hospital. The control group comprised 13 males and 7 females with

an age range between 45 and 60 years (mean = 53.15 ± 4.34). Individuals with liver disease, kidney disorders or risk factors for atherosclerosis other than diabetes, such as a body mass index (BMI) >30 , dyslipidaemia, hypertension or smoking, were excluded from the study. All patients and controls provided a full clinical history and underwent: i) a full clinical examination including measurement of blood pressure and waist circumference, as well as a cardiac and abdominal examination, ii) ultrasonographic scanning of the intima/media thickness (IMT) of their carotid arteries (Doppler ultrasonography), iii) routine laboratory investigations [fasting and 2-h postprandial plasma glucose, complete lipid profile including triglyceride, cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), liver and kidney function tests], and iv) genotyping of the p22 phox gene for the C242T polymorphism by means of polymerase chain reaction (PCR).

Blood samples were collected from patients and controls and divided into two aliquots. One was used for routine investigations, and the other was placed in an EDTA-containing tube for use in genomic DNA extraction and genotyping of the p22 phox gene.

p22 phox gene genotyping for the C242T polymorphism. The genotype of the p22 phox gene (C242T polymorphism at exon 4) was investigated using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Genomic DNA was extracted from whole peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was then used as a template to amplify exon 4 of the p22 phox gene using the primers F: TGCTTGTTGGTAAACCAAGGCCGGTG, and R: AACACTGAGGTAAGTGGGGGTGGCTCCTGT (12). The reaction mixture was performed in a 50- μ l final volume containing 0.5 μ g extracted DNA, 200 μ M of each primer and 25 μ l Taq PCR master mix (2.5 units Taq DNA polymerase, 1X PCR buffer and 200 μ M of each dNTP; Taq PCR Master kit, Qiagen). PCR conditions included initial denaturation at 94°C for 5 min followed by 35 cycles of 50 sec denaturation at 94°C, 50-sec of annealing at 60°C and a 1-min extension at 72°C. This was followed by a final extension at 72°C for 7 min (Applied Biosystem 5700 thermal cycler). The PCR product was first digested by the *rhodobacter sphaeroides* I (*Rsa*I) restriction enzyme, fractionated on 3% agarose gel containing ethidium bromide, and visualized by UV illumination. Digestion of the PCR products yielded bands of 348 bp in the CC homozygote, 188 and 160 bp in the TT homozygote, and all three bands in the heterozygotes (13).

Duplex scanning of the carotid artery. The carotid arteries were examined with the subject in the supine position and the examiner seated at the subject's head. Exposure of the neck was maximized by having the subject drop the ipsilateral shoulder as far as possible and rotating the head away from the side being examined. The wall of the carotid artery was thus exposed, producing two parallel echogenic lines corresponding to the adventitia and the intimal layers of the arterial wall, with the intervening hypoechoic layer representing the media and the intimal reflection straight, thin and parallel to the adventitial layer. Significant undulation or thickening of

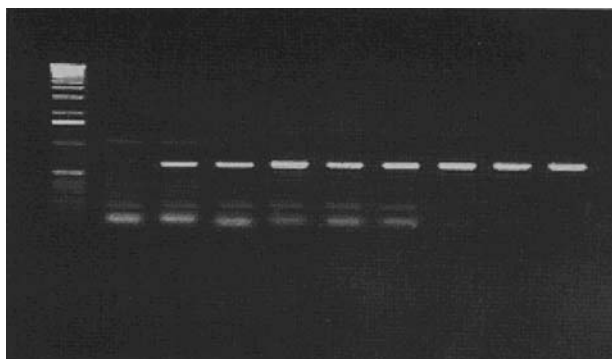


Figure 1. Agarose gel electrophoresis showing the product of *RsaI* RFLP of the p22 phox gene. Lane 1, 1-kb DNA ladder; lane 2, two DNA bands (150 and 188 bp) representing the homozygote of the TT genotype; lanes 3-7, three DNA bands (160, 188 and 348 bp) representing the heterozygote of the TC genotype; lanes 8-10, one DNA band (348 bp) representing the homozygote of the CC genotype.

the intima was taken to indicate plaque deposition. Values under ≤ 1.1 mm were considered to be normal IMT, while values > 1.1 mm were considered to be abnormal, and a possible indicator of future atherosclerotic morbidity (12).

Statistical analysis. Data were collected, presented and statistically analyzed by the SPSS computer program, version 11, using the following tests: i) the ANOVA test to compare the means of grouped observations, ii) the χ^2 test to test for differences in proportion, iii) the unpaired T-test to compare the means of the two groups, and iv) the linear regression test for correlation. If (r) was a positive fraction then the two variables tended to increase or decrease together. If (r) was a negative fraction then one variable increased as the other decreased. P-values < 0.05 were considered statistically significant (14).

Results

Frequencies of the p22 phox C242T genotypes. The frequency of the C allele of the CYBA gene at position 242 was 71.7%

(CC), and that of the T allele 28.3% (TC+TT). Genotype frequencies were as follows: CC, 71.7; TC, 21.7; TT, 6.6% (Fig. 1).

Relationship between clinical characteristics and the C242T genotype. To analyze the effect of the C242T polymorphism of the p22 phox encoding gene as a risk factor for atherosclerosis, patients were divided into two groups depending on their p22 phox C242T genotype. One group had the C allele at position 242 (homozygous CC genotype) and the other had the T allele at position 242 (homozygous TT and heterozygous TC). The two groups were compared statistically in terms of age, gender, risk factor for atherosclerosis and carotid artery IMT, both to each other and to the control group. No significant difference was found between group 1 and group 2 regarding the duration of DM, fasting and 2-h postprandial blood glucose, or glycated hemoglobin levels ($P=1.644$, $P=0.460$, $P=0.553$ and $P=0.810$, respectively).

As shown in Table I, with the exception of carotid IMT, there was no association between any of the clinical features studied and the p22 phox C242T genotype. No significant difference was found among the three groups in terms of age ($P=1.828$), gender ($P=0.141$), BMI ($P=0.363$), total serum cholesterol ($P=0.142$), triglycerides ($P=0.68$), HDL-C ($P=0.122$) or LDL-C ($P=0.176$). Similarly, blood pressure, both systolic and diastolic, did not differ significantly between the three groups ($P=0.107$ and $P=0.284$ for systolic and diastolic blood pressures, respectively).

Relationship between carotid artery intima/media thickness and the p22 phox C242T polymorphism. The IMT of the carotid artery was measured by color duplex scanning, and the results for all subjects were statistically analyzed. An IMT > 1.1 mm was considered to be abnormal, and an increase in the carotid IMT was significantly associated with the absence of the T allele at position 242 of the p22 phox gene. As shown in Table I, carotid IMT differed significantly between the three groups ($P<0.0001$). The carotid IMT of diabetic patients in group 1, lacking the T242 allele (mean IMT 1.36 ± 0.177 mm),

Table I. Clinical characteristics of patients with and without the T allele at position 242 of the p22 phox gene.

Clinical features	Group 1 C242C (n=30)		Group 2 T242C and T242T (n=10)		Control group (n=20)		P-value
	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	
Age (years)	45-59	51.1 \pm 3.83	45-60	53.2 \pm 4.89	45-60	53.15 \pm 4.34	1.8280
Gender (M/F) (number)	18/12		6/4		13/7		0.1410
BMI (kg/m ²)	23.35-28.91	26.23 \pm 1.35	24.8-27.61	25.988 \pm 0.946	24.11-28.66	26.638 \pm 1.302	0.3630
Total cholesterol (mg/dl)	178-210	193.7 \pm 8.73	175-205	189.7 \pm 9	170-205	188.7 \pm 9.63	0.1420
Triglyceride (mg/dl)	89-160	122.6 \pm 23.6	85-155	117.5 \pm 24.8	80-155	117.1 \pm 23.8	0.6800
HDL-C (mg/dl)	36-48	40.9 \pm 3.3	39-49	42.6 \pm 3.17	38-52	42.9 \pm 3.88	0.1220
LDL-C (mg/dl)	116-152	128.1 \pm 10.4	113-147	123.6 \pm 11.3	105-147	122.3 \pm 12.4	0.1760
SBP (mmHg)	110-135	122.6 \pm 7.7	110-130	118.5 \pm 7.6	105-135	118.5 \pm 7.6	0.1070
DBP (mmHg)	70-85	80.8 \pm 4.5	70-85	79 \pm 5.1	65-85	78.5 \pm 6.3	0.2840
IMT (mm)	0.98-1.63	1.36 \pm 0.177	0.85-1.25	1.033 \pm 0.143	0.72-0.96	0.828 \pm 0.072	<0.0001

BMI, body mass index; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure, IMT, intima/media thickness.

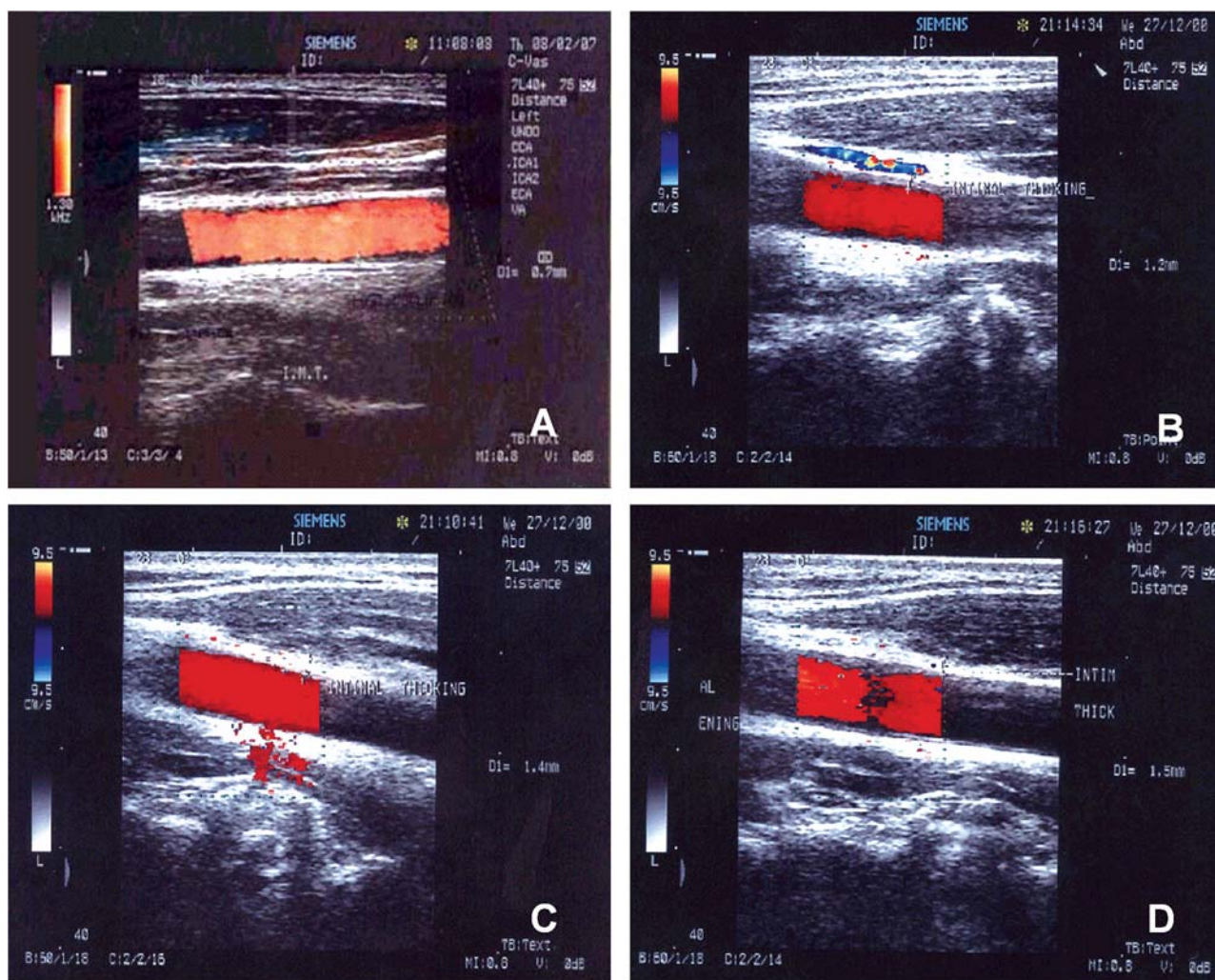


Figure 2. Colored duplex showing the IMT of carotid arteries. (A) Carotid IMT measuring 0.7 mm in a control subject, (B) carotid IMT measuring 1.2 mm in a patient with the TC genotype, (C) carotid IMT measuring 1.4 mm in a patient with the CC genotype, (D) carotid IMT measuring 1.5 mm in a patient with the CC genotype.

and in group 2, with a homozygous or heterozygous 242T allele (mean IMT 1.033 ± 0.143 mm), was significantly greater than that of the control group (mean IMT 0.828 ± 0.072 mm) ($P < 0.0001$). Similarly, the carotid IMT in group 1 diabetic patients, who lacked the 242T allele (C242C genotype), was significantly greater than in group 2 diabetic patients, who had the 242T allele (T242C and T242T) ($P < 0.0001$). In non-diabetic subjects, the average carotid IMT of the TC+TT group did not differ from that of the CC group (0.81 ± 0.08 vs. 0.84 ± 0.069 mm, $P > 0.05$) (Fig. 2).

The association study revealed a significant positive correlation between carotid IMT and the duration of DM, HbA1c and blood glucose level, as well as a significant negative correlation between carotid IMT and presence of the 242T allele in the p22 phox gene.

Discussion

The chronic hyperglycemia of DM is associated with long-term damage, dysfunction and the failure of various organs, in particular the eyes, kidneys, nerves, heart and blood vessels (4). The prevalence of macrovascular disease is markedly increased among individuals with DM. Its major clinical

manifestations are atherosclerosis of the coronary arteries, cerebral arteries and large arteries of the lower extremities (15).

Oxidative stress and increased O_2^- production are key features of the vascular phenotype in human atherosclerosis, affecting nitric oxide scavenging, peroxynitrite generation and the redox-sensitive cell signaling pathways. The NAD(P)H oxidase enzyme is an important source of O_2^- in human vessels. The activity of this enzyme system is increased in association with an atherosclerotic risk factor profile, and is more marked with diabetic hyperglycemia. NAD(P)H oxidase is a multi-subunit enzyme complex. One of its subunits is the p22phox protein, a key component of the enzyme required for NAD(P)H oxidase activity and for the production of O_2^- (16). Genetic factors including polymorphisms of the CYBA gene, the gene which encodes p22phox, modulate NAD(P)H oxidase activity and, therefore, O_2^- production. This leads to significant variations in vascular oxidative stress between individuals, and in turn modulates susceptibility to atherosclerosis (11).

The present study aimed to identify the relationship between the C242T polymorphism in the gene encoding NAD(P)H oxidase, the p22phox subunit and carotid IMT in Egyptian type 2 diabetic patients. It was demonstrated that the frequencies of the C and T alleles at position 242 of the

CYBA gene are similar to international frequencies. The C242C homozygous genotype was the most frequent, followed by heterozygous T242C. Least frequent was the homozygous T242T genotype. Similar results were reported by Hayaishi-Okano *et al* (17).

In order to demonstrate the effect of the p22 phox C242T polymorphism on the risk of atherosclerosis in diabetic patients, we measured the IMT of the common carotid artery and analyzed the data in relation to patient genotype. Patients were classified into two groups according to the presence or absence of the T allele (CC vs TC/TT). To reduce the effect of other risk factors for atherosclerosis on analysis, patients in both groups were chosen in such a way that no significant differences existed between them in terms of these factors, and were statistically matched in terms of blood glucose level, HbA1c, duration of diabetes, BMI and lipid profile.

The study demonstrated a significant increase in the carotid IMT of diabetic patients compared to healthy controls. These findings are in agreement with those of Tomasz *et al* (18), who found that carotid atherosclerosis is more prevalent among diabetics, and observed that the vessels of patients with DM generate significantly more free radicals than those of non-diabetics, accelerating the progression of atherosclerosis. These free radicals are produced due to the increased concentration and activity of the vascular NAD(P)H oxidase system in diabetes. Additionally, in diabetic vessels, the endothelium is a net contributor to total vascular O_2^- release rather than to the scavenging of O_2^- by nitric oxide production. Increased endothelial O_2^- production appears to be caused by dysfunctional endothelial nitric oxide synthase (eNOS) mediated by the loss of cofactor BH_4 availability. Similarly, Lee *et al* (19) observed an increased rate of progression of carotid atherosclerosis in type 2 diabetes.

Genetic polymorphisms within the promoter or the coding sequence of the CYBA gene, which encodes p22phox, may have a strong influence on gene expression, enzyme activity and vascular O_2^- generation. This leads to significant functional variation between individuals in terms of vascular oxidative stress and nitric oxide bioactivity, and hence in their liability to atherosclerosis (16). Our findings show that the C242T polymorphism of the p22 phox gene is associated with significant changes in carotid IMT in diabetic patients. Diabetic subjects with the 242T allele (TC/TT genotype) displayed a significantly lower average of carotid IMT than those lacking the 242T allele (CC genotype) ($P < 0.0001$), despite the fact that there was no difference in the other risk factors for atherosclerosis between the two groups. This finding means that the 242T allele could be considered protective, while the CC genotype could be considered a risk factor for atherosclerosis in diabetics. Similar results have been reported by Inoguchi *et al* (20), who moreover observed significantly lower O_2^- production in the vessels of diabetic patients with the 242T allele, independently of other atherosclerotic risk factors. Hayaishi-Okano *et al* (17) reported that a lower carotid IMT was associated with the CYBA 242T allele, and that diabetic Japanese with the CC genotype had increased activated NAD(P)H oxidase through the PKC pathway, while 242T diabetic carriers were associated with significantly reduced enzyme activity and ROS production. One possible mechanism is that the C242T polymorphism of the p22 phox gene

results in the replacement of histidine by tyrosine at amino acid position 72 in the p22phox subunit. This leads to a reduction in NAD(P)H oxidase activity and a decrease in O_2^- production, which in turn decreases susceptibility to atherosclerosis. The absence of differences in carotid IMT between the CC and TC/TT genotypes of normal subjects indicates that the risk effect of the CC genotype is significant only in the presence of other risk factors, such as hyperglycemia.

In contrast, Cai *et al* (21) had conflicting results, and reported that the CYBA 242T allele was associated with the increased progression of atherosclerosis in Australian patients, but not in the overall population. The mechanism underlying the association of the 242T allele with the progression of atherosclerosis is unknown. However, the disparities in results could be related to differences in the ethnicity of the patient populations.

DM is an independent risk factor for increased carotid IMT, and hence of atherosclerosis. The present study demonstrated a positive correlation between carotid IMT and blood glucose level and duration. Patients with uncontrolled DM tend to have greater carotid IMT, as do those with higher blood glucose levels, higher HbA1c and longer duration of DM. In agreement with these results, the Northern Europe research group demonstrated a direct correlation between blood glucose control and cardiovascular events and mortality (22). The primary casual factor in the pathogenesis and development of atherosclerosis is prolonged exposure to hyperglycemia, which mediates its effects in several different ways. Glucose in sustained high concentrations may be directly toxic to cells, thus altering cell growth and protein expression and increasing extracellular matrix and growth factor production (3).

In conclusion, the present study demonstrated a significant association between the C242T polymorphism of the p22 phox gene, which encodes an important subunit of NAD(P)H oxidase, and carotid IMT in Egyptian type 2 diabetic patients. The C242C genotype was associated with a significant increase in carotid IMP, and consequently with the enhancement of atherosclerosis, while the presence of the 242T allele was associated with a significantly reduced carotid IMT. Therefore, the 242T allele of the p22 phox gene could be considered a protective factor against atherosclerosis in diabetic patients.

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