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Endothelial Nitric Oxide Synthase Gene Polymorphism and Risk of Coronary Artery Diseases in Egypt

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***Abstract*:** *The present study aimed to investigate the association of endothelial nitric oxide synthase gene polymorphism with susceptipility and severity of coronary artery diseases (CAD) in Egyptian cases. The study included 100 cases from intensive care units (ICU) of Cardiology Department of Internal Medicine, University Hospital, as well as Ministry of Health Hospitals of Dakahlia (Mansoura University Hospital) & Gharbia (Elmehalla Heart Center) governorates ,divided into 50 patients with coronary artery diseases and 50 healthy as controls .The mean age of cases was 57.28±11.51, including 40 males and 10 females .Blood samples were collected in tubes for biochemical analysis (5 ml) from each case {Hb concentration, W.B.Cs, AST, ALT, creatinine, cholesterol, TG, LDL, HDL, LDH}. DNA was amplified using PCR-SSP for detection of relation between polymorphism and endothelial nitric oxide synthase gene in three parts G894T, T786C and 27 bP. All cases showed significant differences between cases and controls regarding their chemical lab's analysis {Hb concentration,W.B.C.s, AST, ALT, creatinine, cholesterol, TG, LDL, HDL, LDH} .All cases showed significant frequency of G894T GG(p> 0.05 ,OR =0.49) and G894T TT (p=0.001 ,OR =0.37). Also there were significant frequency of T786C TT (p>0.05, OR=0.57) .C786T CC(P=0.004 ,0R=4.58) ,and there were no significant frequency of 27bp aa (p=0.032 ,OR=2.891) ,27bp bb (p>0.05, OR =1.86). These were considered risk genotypes for diseases susceptibility. On the other hand all cases showed no significant frequency with combined heterozygosity for G894T GT (P>0.05, OR=0.67) or C786T CT(P>0.05 ,OR =0.72) or 27bp ab (p=0.001, OR=0.25). So , endothelial nitric oxide synthase gene polymorphism in G894T andT786C can be considered genetic markers for coronary artery diseases among Egyptian cases.*

Keywords: *coronary artery disease, risk factors polymorphism, gene, endothelial nitric oxide synthase.*

1. Introduction

Coronary artery diseases (CAD) are the leading cause of cardiovascular related deaths worldwide. Multiple risk factors including age, sex, smoking, hypertention, diabetes and genetic predisposition influence the onset of CAD (Faxon et al., 2004 & puddu et al., 2005).

Atherosclerosis, a prerequisite for the development of CAD, results from a defective endothelial function, which is attributed mainly to an altered production of nitric oxide(NO), a vasodilator and athero protective molecule (Davignon and Ganz, 2004).

Nitric Oxide is synthesized via a reaction that included the conversion of L-arginine to L-citruline catalyzed by endothelial nitric oxide synthase (eNOS), which is one of the three isoforms of the enzyme (Mayer and Hemmens, 1997).

Endothelial nitric oxide synthase is the product of eNOS gene , which is 21 Kb in size and consists of 26 exons (Marsden et al., 1993). Additionally, promoter region of the gene harbors several transcription factor binding sites, regulating gene expression because eNOS availability is regulated at transcriptional and

posttranscriptional levels and owing to its role in the production of NO. This gene is considered to be a potential candidate for cardiovascular diseases (Searles, 2006).

Accordingly several eNOS gene variants including single nucleotide polymorphisms(SNPs), a variable number of repeats in the intron 4 and a cytosine adenine (CA) repeat microsatellite marker in the intron 13 (Wang et al., 1996 & Hingorani et al., 1999).

Additionally, sequence variations have also been reported in the promoter region of eNOS gene (Nakayama et al,. 1999).

Among the known eNOS polymorphisms, the most examined and functionally related common variants are G894T , T786C and 27bP repeat (Litwack, 2014).

2. Subjects and Methods

The study included 100 cases from intensive care units (ICU) of Cardiology Department of Internal Medicine, University Hospital, as well as Ministry of Health Hospitals of Dakahlia (Mansoura University hospital) & Gharbia (Elmehalla Heart Center) governorates ,divided into 50 patients with coronary artery diseases and 50 healthy as controls , The mean age of cases was 57.28±11.51, including 40 males and 10 females.

Venous blood samples (3ml) were collected from each case and control in poly ethylene tubes containing ethylene diamine tetracetic acid (EDTA) with pH 8.0 as an anticoagulant. From some of this anticoagulated blood (200µ1), DNA was extracted from peripheral blood using a rapid non-enzymatic method determined by (Lahiri and Nurnberger, 1991), purified, amplified and analyzed .

Hemoglobin and WBCs count were evaluated using automated blood counter device (mindray BC-2800).

AST, ALT and LDH enzymes were evaluated using quantitative method (Biomed kits).

Createnine was evaluated using quantitative method (Biomed kit).

Blood sample (5 ml) from all cases and controls were obtained in the morning after fasting for 12 hours. They were collected in glass tubes and allowed to clot at room temperature then ised for the determination of serum lipids. Immediately following clotting , serum was separated by centrifugation for 15 minutes at 3000 rpm. The levels of TC, TG, HDL-C and LDL-C in samples were determined.

Serum total cholesterol was determined according to the method of (Allain et al,. 1974) using kits of, Linear Chemicals, S.L (Spain) that is involved the use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidise(POD). In the presence of the former mixture of phenol and 4-aminoantipyrine (4-AA) condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in sample.

3. Statistical Analysis

Data were processed and analyzed using the statistical package of social science (SPSS, version 10.0). The frequency of studied allelic polymorphism among cases were compared to that of control describing number and percent of each and tested for positive association using Fisher's exact test (modified Chi square test) and Odds with a minimum level of significance of < 0.05

4. Results

On studying general characteristics of cases of coronary artery diseases and healthy controls (table1): the mean age (±SD) and the gender frequency in cases of coronary artery diseases were non-significantly different from that of the controls as the mean standards for age were (55.82 ± 8.96, 57.28 ± 11.51) for healthy controls and cases of CAD respectively and P value 0.115 (males were 33 while females were 17 in healthy controls and males were 40 while females were 10 in cases of CAD).

However the mean value (±SD) of Hb concentration, WBCs, AST, ALT, Createnine and LDH were significant different ($p < 0.05$ for each) (12.71 ± 1.67), (9.13 ± 2.46), (68.96 ± 78.32), (39.52 ± 20.94), (1.07 ± 0.34), (1.07 ± 0.34) and (923.98 ± 733.05) for cases of CAD respectively than in healthy controls (13.44 ± 1.93), (5.45 ± 1.35), (30.00 ± 6.89), (31.56 ± 8.22), (0.66 ± 0.26) and 240 ± 40 respectively.

While the mean values (±SD) of cholesterol, TG, LDL-C and HDL-C were highly significant ($p < 0.001$ for each) (205.60 ± 58.15), (154.10 ± 79.15), (124.74 ± 43.32) and (49.42 ± 11.85) for cases of CAD respectively than in healthy controls (83.16 ± 16.50), (78.40 ± 14.83), (83.88 ± 11.10) and (60.96 ± 7.03) respectively.

Comparing all cases with CAD and healthy controls regarding their genotype distribution of eNOS gene polymorphism [in G894T] (table2): only (TT) genotype was highly significant (p=0.001), (32% of cases vs. 6% of controls) . While on allele was higher among controls (68% of controls and 47% of cases) but (T) allele was higher controls among cases (53% of cases and 32% of controls).

Comparing all cases with CAD and healthy controls regarding their genotype distribution of eNOS gene polymorphism [in T786C] (table3): only (CC) genotype was highly significant (p=0.004), (24% of cases vs. 4% of controls). While allele analysis both (T) and (C) were significant ($p \leqslant 0.05$ for both) and (T) allele was higher among controls (67% of controls and 51% of cases) but (C) allele was higher among cases (49% of cases and 33% of controls).

Comparing all cases with CAD and healthy controls regarding their genotype distribution of eNOS gene polymorphism [in 27 bp repetition] (table4): both genotypes (aa) and (ab) were significant where (aa) genotype was higher among cases (p=0.032) and (ab) genotype higher among healthy controls (p=0.001) while (bb) genotype and allele (a and b) analysis didn't show any significant difference between both.

Wild type GG appears at 206 b only lanes 4 and 6 . digestion of PCR product of G894T polymorphism of eNOS gene using Mbo1 enzyme , which digests the 206-bp fragment into 119 and 87 bp fragments (heterozygous mutated genotype GT which has 206 , 119,87 bp fragments lanes 2 and 7) but (homozygous mutated genotype TT is found which has 119, 87 bp fragments lanes 1,3,5) by using DNA size marker 50bp.(Fig1)

Wild type TT appears at 236bp in lanes 1,2,3&5 digestion of PCR product of T786C polymorphism of eNOS gene using NgoMIV enzyme which digests the 236-bp fragment into 203 and 33-bp fragments (heterozygous mutated genotype TC which has 236, 203, 33 bp fragments lanes 6 only) but (homozygous mutated genotype CC is found which has 203, 33 bp fragments lanes 4,7) by using DNA size marker 50 bp (Fig2).

Electrophoresis result of PCR showing PCR amplification of intron 4b/a polymorphism of eNOS gene PCR product of intron 4b/a polymorphism has ban size (220) bp in bb carrier homozygous and ba carrier heterozygous which has (220,193bp fragments bane 2 and 4) by using DNA marker 50 bp . (Fig3).

TABLE I: Descriptive Data of Studied Cases of Coronary Artery Diseases and Healthy Controls

	Controls (n = 50)	Cases (n = 50)	P
Age (years)			
Age range	39 – 79	25 – 85	
Mean age ± SD	55.82 ± 8.96	57.28 ± 11.51	0.481
Sex: Male Female	 33 17	 40 10	0.115
Hb (gm/dl)	13.44 ± 1.93	12.71 ± 1.67	0.046*
W.B.Cs (U/L)	5.45 ± 1.35	9.13 ± 2.46	< 0.001**
AST (IU/L)	30.00 ± 6.89	68.96 ± 78.32	< 0.001**
ALT (IU/L)	31.56 ± 8.22	39.52 ± 20.94	0.015*
Creatinine (mg/dl)	0.66 ± 0.26	1.07 ± 0.34	< 0.001**
Cholesterol (mg/dl)	83.16 ± 16.50	205.60 ± 58.15	< 0.001**
T.G (mg/dl)	78.40 ± 14.83	154.10 ± 79.15	< 0.001**
LDL-C (mg/dl)	83.88 ± 11.10	124.74 ± 43.32	< 0.001**
HDL-C (mg/dl)	60.96 ± 7.03	49.42 ± 11.85	< 0.001**
LDH (IU/L)	240 ± 40	923.98 ± 733.05	< 0.001**
Consanguinity			
Positive	0 (0%)	9 (18%)	0.002*
Negative	50 (100%)	41 (82%)	
Family history			
Positive	0 (0%)	30 (60%)	< 0.001**
Negative	50 (100%)	20 (40%)	

Hb = hemoglobin , w.b.cs = white blood cells count, AST = Serum glutamic oxaloacetic transaminase , ALT= Serum glutamic pyruvic transaminase, TC= total cholesterol, LDL-C = low-density lipoprotein , HDL-C = high-density lipoprotein , TG= triglyceride, LDH=Lactate dehydrogenase, n = number of cases

Significance using t-test or Chi square test :

* $p \leq 0.05$ (significant).

** $p \leq 0.001$ (extremely significant)

TABLE II: Comparison between all Cases with CAD and Healthy Controls Regarding Their Genotype Distribution of eNOS Gene Polymorphism in G894T

G894T		Controls n (%)	Cases n (%)	P
Genotypes	(GG)	21 (42%)	13 (26%)	> 0.05
	(GT)	26 (52%)	21 (42%)	> 0.05
	(TT)	3 (6%)	16 (32%)	0.001**
Alleles	(G)	68 (68%)	47 (47%)	< 0.05*
	(T)	32 (32%)	53 (53%)	< 0.05*

n=number of cases, (%)= percentage of cases, OR(95% CI) = odds ratio & 95 % confidence interval , GG = guanine guanine , GT= guanine thymine ,

TT= thymine thymine , G= guanine , T= thymine

Significance using Fisher's Exact test: * $p \leq 0.05$ (significant).

** $p \leq 0.001$ (extremely significant).

TABLE III: Comparison between all Cases with CAD and Healthy Controls Regarding their Genotype Distribution of eNOS Gene Polymorphism in T786C

T786C		Controls n (%)	Cases n (%)	P
Genotypes	(TT)	19 (38%)	13 (26%)	> 0.05
	(TC)	29 (58%)	25 (50%)	> 0.05
	(CC)	2 (4%)	12 (24%)	0.004*
Alleles	(T)	67 (67%)	51 (51%)	< 0.05*
	(C)	33 (33%)	49 (49%)	< 0.05*

n=number of cases, (%)= percentage of cases, OR(95% CI) = odds ratio & 95 % confidence interval , TT = thymine thymine , CT = cytosine thymine, CC = cytosine cytosine , C = cystosine , T = thymine

Significance using Fisher's Exact test: * p≤ 0.05 (significant).

TABLE IV: Comparison between all Cases with CAD and Healthy Controls Regarding their Genotype Distribution of eNOS Gene Polymorphism in 27 bp Repetition

27bp		Controls n (%)	Cases n (%)	P
Genotypes	(aa)	7 (14%)	16 (32%)	0.032*
	(ab)	29 (58%)	13 (26%)	0.001**
	(bb)	14 (28%)	21 (42%)	>0.05
Alleles	(a)	43 (43%)	45 (45%)	>0.05
	(b)	57 (57%)	55 (55%)	>0.05

n=number of cases, (%)= percentage of cases, OR(95% CI) = odds ratio & 95 % confidence interval , a= allele a , b=Allele b

Significance using Fisher's exact test:

* p≤0.05 (significant).

** p ≤0.001 (extremely significant).

Fig. 1: Enzymatic digestion of G894T polymorphism of eNOS gene

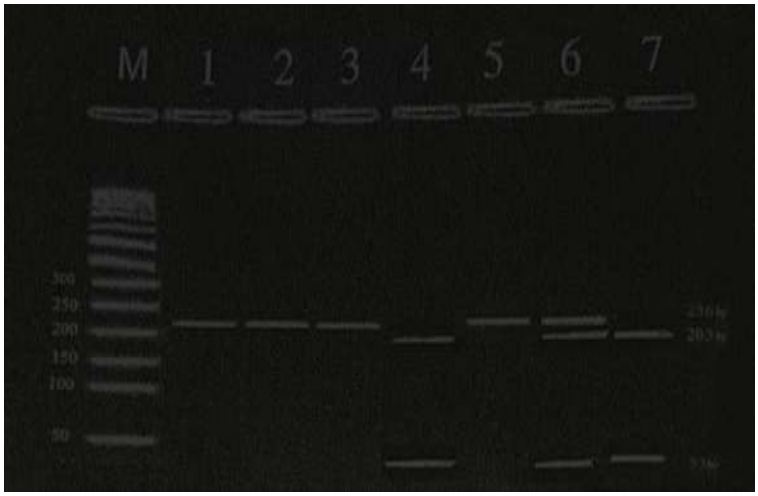

Fig. 2: Enzymatic digestion of T786C polymorphism of eNOS gene.

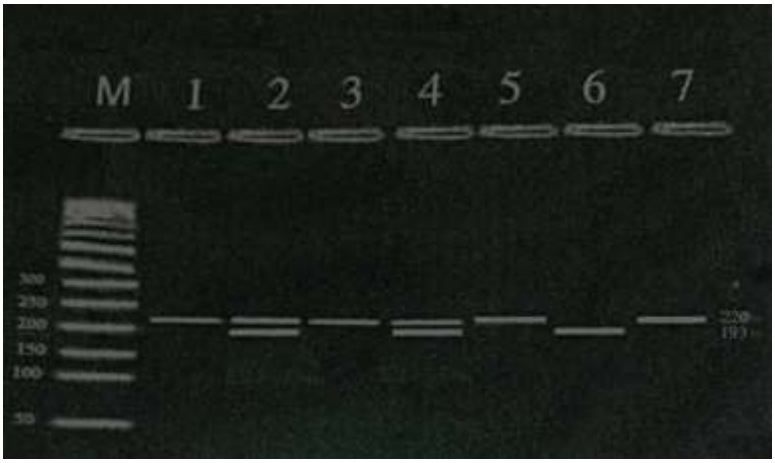

Fig 3: PCR amplification of intron 4b/a polymorphism of eNOS gene

5. Discussion

Coronary artery diseases (CAD) are multifactorical diseases which cause the greatest mortality and morbidity of many diseases in the world. Along with recognized independent risk factors for CAD such as obesity , hypertension, diabetes mellitus, hypercholesterolemia ,sex family history and smoking, it is suggested that a specific genetic predisposition should be taken into account(Berdeli et al.,2005).

NO is a primary physiological transmitter derived from the endothelium, which plays a composite role with such diverse antiatherogenic effects as vasodilator (Vanin, 1998) and antioxidant in the walls of the blood vessels. NO increases intracellular cGMP by stimulating soluble guanylate cyclase, resulting in the relaxation of vascular smooth muscle cells mediating vasodilatation (Prabhakar et al.,1998).

It also inhibits platelet and leukocyte adhesion to the endothelium, limits vascular smooth muscle cell migration and growth and acts to inhibit low density lipoprotion(LDL-C) oxidation (Salazar et al., 2000). It has been found that inadequacy or reduction in the bio-avalability of NO in the vascular endothelium contributes to the occurrence of atherosclerosis (Oemar et al., 1998). NO is synthesized from arginine by means of endothelial nitric oxide synthase (eNOS), an isoform of nitric oxide synthase (NOS), which is dominant in the blood vessel walls. A polymorphism in the eNOS gene is one of the genetic risk factors considered to have an important role in the formation of CAD (Sigusch et al.,2000).The present study determined the genotype distribution of the eNOS(G894T,T786C and 27 bp) gene region in CAD cases and their allele frequencies.

There are many reports indicating a significant association between variations of the eNOS gene and the incidence of CAD, a study explored the relationship between polymorphic eNOS alleles and CAD in eastern Taiwan suggested that the G 894T polymorphism in exon 7 of the eNOS gene is likely to be a risk factpr for CAD (Lin et al., 2008).

Another study in Saudi Arabia also agrees with the present results which found that cases of CAD and controls differed significantly in genotype and allel frequencies of G894T polymorphism of eNOS gene. Additionally, this variant is independently associated with CAD even after adjusting for several potential CAD risk factors . Furthermore, genotype frequencies of G894T polymorphism in the studied population are inclined towards Caucasian rather than Asian population (Alkharfy et al., 2010).

Dafni et al., (2010) made a study on Greek population indicated that G894T polymorphism of the eNOS gene seems to be associated with myocardial infraction (MI) occurrence in their population . They found evidence that hpmpzygous TT is positively related to the risk of MI and this association is independent of possible effects of other known MI risk factors. They found also that smokers , hypertensive and those with a family history of CAD were more likely to develop MI.

Data obtained from a study on Algerian population found association between G894T polymorphism and acute myocardial infarction but this study was not effective enough to confirm that the eNOS gene could represent useful genetic markers for identifying individuals at risk for development of CAD (Meroufel et al., 2009).

Mahmoodi et al. (2016) study carried on 200 patients with angiographically documented CAD and 100 matched controls .Analysis of G894T genetic polymorphism of eNOS found that reduced plasma level of NO was associated with increased risk of CAD in Iranian population. Moreover, eNOS G894T polymorphism was a significant risk factor for CAD development via reducing the plasma levels of NO . However, eNOS G894T polymorphism was not a contributing factor for the severity of CAD.

A study carried in Tehran Shahid Rajaee Heart Hospital, Salimi et al., (2012) found that genotype frequencies of T786C polymorphism in promoter were differed significantly between CAD cases and controls and the frequency of the C allele of the T786C polymorphism was significantly higher in CAD patients than in controls and thus agrees with us.

Another study by Hoogeveen et al., (2014) investigated the relationship between plasma levels of small dense low-density lipoprotein-cholesterol and risk for incident CAD in a prospective study among Atherosclerosis Risk on Communities (ARIC) study participants, they found that sdLDL-C was associated with incident CHD with a hazard ratio of 1.51 (95% confidence interval, 1.21-1.88) for the highest versus the lowest quartile, respectively. Even in individuals considered to be at low cardiovascular risk based on their LDL-C levels, sdLDL-C predicted risk for incident CHD (hazard ratio, 1.61;95% confidence interval, 1.04-2.49).

6. Conclusion

G894T and T786C polymorphism of eNOS gene was found to be associated with development of coronary artery diseases and mutant G,T and T,C (GG and TT genotypes pf G894T) and (TT and CC genotypes of T786C) maybe considered genetic risk factors for development of coronary artery diseases in Egypt. But we can't be sure that 27 bp repeat considered risk factor as there was no significant difference between cases and controls.

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