Endothelial nitric oxide synthase Gene Polymorphism (G894T) in coronary artery disease in Egyptian patients

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Abstract: Background: Endothelial nitric oxide synthase (eNOS) could be a candidate gene for coronary artery disease (CAD). **Objectives:** To check for the association of polymorphisms of Endothelial nitric oxide synthase (eNOS) (G894T) gene with the susceptibility and severity of coronary artery disease in Egyptian patients. **Subjects:** This work included 70patient with coronary artery disease and 62 healthy individuals. The mean age of cases was 60.68± 11.29 years (range: 35.00-94.0 years). They included 36 males and 34 females. **Methods:** DNA was amplified using PCR-SSP for detection of polymorphisms related to endothelial nitric oxide synthase (G894T) gene. **Results:** Total cases showed significant frequency of G894T GG (*P*=0.039, OR=0.476), G894T TT (*P*=0.001, OR=7. 327). These were considered risk genotypes for disease susceptibility. On the other hand, total cases showed non significant frequency with combined heterozygosity for G894T GT **(***P***=** 0.546, OR=0.784**)**. **Conclusions:** Polymorphisms related to endothelial nitric oxide synthase G894T gene may be considered as genetic markers for coronary artery disease among Egyptian cases.

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Keywords: Coronary artery disease, Risk factors, Gene Polymorphism, Endothelial nitric oxide synthase**.**

Abbreviations: Coronary artery disease (CAD), Endothelial nitric oxide synthase (eNOS), Polymerase chain reaction with sequence specific primers (PCR-SSP), Restriction fragment length polymorphism (RFLPs) .

1. Introduction

Coronary artery disease (CAD) is the leading cause of cardiovascular-related deaths worldwide. Multiple risk factors including age, sex, smoking, hypertension, 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 atheroprotective molecule **(Davignon and Ganz ,2004).**

NO is synthesized via a reaction that includes 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)**.

The eNOS 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 eNOS gene harbors several transcription factor binding sites,for regulating gene expression.The eNOS availability is regulated at transcriptional and post transcriptional levels and owing to its role in the production of NO , eNOS 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 tandem repeats in the intron 4 and a cytosine adenine (CA) repeat microsatellite marker in the intron 13 (**Wang *et al.*, 1996 *;* Stangl *et al.*, 2000).** Additionally, sequence variations have also been reported in the promoter region of the eNOS gene **(Nakayama *et al.*, 1999*)*.**

The reported variants of the eNOS gene, single nucleotide polymorphism (SNP) in the promoter region G to T trans version at 894 position in exon 7 (G894T), which results in the incorporation of aspartate in place of glutamate (Glu298Asp), are widely studied and found to be associated with low plasma NO concentrations and reduced vascular reactivity, emphasizing their importance in the onset of CAD **(Asakimori *et al.*, 2003*)***. A number of studies have found G894-T polymorphisms of eNOS gene to be associated with the risk of developing cardiovascular diseases either independently or through gene/environmental interactions **(Nasreen *et al.*, 2002; Asakimori *et al* ., 2003; Rossi *et al* ., 2003;Hassan *et al*., 2004; Tangurek *et al* ., 2006;Kim *et al* ., 2007).** Whereas in contrast,

several studies have found the lack of such associations **(Granath *et al* ., 2001;Fatini *et al* ., 2004, Fatini *et al* ., 2004; Jaramillo *et al*., 2006;Jaramillo *et al*., 2008; Jaramillo *et al* ., 2009; Meluzı´n *et al* ., 2009).** Thus,the aim of our work was to examin the distribution of G894-T polymorphisms of eNOS gene in Egyptian CAD patients and normal controls. Additionally, we studied the association of these polymorphisms with the incidence of CAD.

2. Subjects and Methods

This study included 70 cases with coronary artery disease recruited from intensive care units (ICU) of Cardiology Department of Internal Medicine, University Hospital, Mansoura University as well as Ministry of Health Hospitals of Dakahlia governorate, Egypt. They comprised 36(51.4 %) males and 34(48.6 %) females with an age ranging between 35-94years and a mean ±SD of 60.68± 11.29 years. 15(21%) Of these cases, had a positive parental consanguinity and 14(20%) had a positive family history of coronary artery disease. The cases genotypes were compared to 62 healthy volunteers from the same localities.

DNA extraction and purification

After obtaining informed consent from all cases and controls, venous blood samples (3 ml) were collected on EDTA (ethylene diamine tetra acetate) containing tubes, DNA was extracted promptly using DNA extraction and purification kit (Gentra Systems, USA) according to manufacturer's instructions and then stored at -20 ^{0} C till use.

PCR amplification

We genotyped one single nucleotide polymorphisms (SNPs) for nitric oxide synthase gene (eNOS) in this case-control study; G894T polymorphisms using polymerase chain reaction with sequence-specific primers (SSP-PCR).

PCR amplification was performed in single SSP-PCR reaction employing a forward and a reverse primer for G894T polymorphisms of eNOS gene. The regions containing one RFLPs within the eNOS gene was amplified with Taq DNA polymerase. PCR Master Mix (2X) is an optimized ready-to-use PCR mixture of Taq DNA Polymerase, PCR buffer, MgCl2 and DNTPs.

Detection of amplified products

The entire reaction volume plus 5 µ1 of bromophenol blue track dye were loaded into 2% agarose gel (Boehringer Mannheim) containing ethidium bromide. Gels were electrophorosed for 30 minutes at 100 V, photographed under UV light (320 nm) and then scored for the presence or absence of an allele specific band.

Statistical analysis:

Data were processed and analyzed using the Statistical Package of Social Science (SPSS, version 10.0). 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 (modified Chi square test).Odds ratio with a minimum level of significance of P <0.05.

3. Results

Analysis of G894T polymorphism (Table 1, Figs. 1-3), showed that Genotype GG is significant in total cases compared to controls (OR=0.476, *P*=0.039). The same was observed for allele G (OR=0.363, *P*=0.01). On the other hand, Genotype TT has shown high significant frequency among total cases (OR=7.327, *P*=0.001). The same was observed for allele T (OR=0.363, *P*=0.01).

Interestingly, the frequency of homozygous mutated TT genotype, of G894T polymorphism of eNOS gene, was nonsignificant among cases with gender of CAD (*P*=0.568), (Table 2). Also, we noted that there was no significant difference among cases of CAD, regarding their genotypes of G894T polymorphism of eNOS gene, when they classified to subgroups according to consanguinity (*P*=0.945), (Table 3).Our data indicate that there was no significant difference between positive and negative family history subgroups of CAD, regarding their genotypes of G894T polymorphism (*P*=0.794),(Table 4).The same results was recorded with no significant difference between positive and negative smokers of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms (*P*=0.401)(Table 5).With the same manner our data indicated that there were no associations between G894T polymorphism and Cholesterol or triglyceride, among subgroups of patients with CAD (*P*=>0.05),(Table 6).On the other side,our study indicated that there was significant difference between Lactate dehydrogenase and G894T polymorphism among subgroups of patients with CAD (*P*= 0.007),(Table 7).While the results indicated that there was no association between G894T polymorphism and liver enzyemes(GPT and GOT)of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms (*P*>0. 05), (Table 8) .

Table (1): Analysis of G894T polymorphism, *P<0.05(significant) using Fisher's Exact test.

	Genotypes			**Alleles**	
	GG n **(%)**	**GT** n **(%)**	**TT** n **(%)**	**G** n **(%)**	**T** n **(%)**
All cases (n=70)	35(50.0)	16(22.9)	19(27.1)	86(61.4)	54(38.6)
H. controls (n=62)	42(67.7)	17(27.4)	3(4.8)	101(81.5)	23(18.5)
P	**0.039***	**0.546**	**0.001****	**0.01***	**0.01***
OR (95% CI)	**0.476(0.234-0.968)**	**0.784(0.356 – 1.726)**	**7. 327(2.049-26.194)**	**0.363(0.206-0.639)**	**0. 363(0.206-0.639)**

Table (2): Comparison between two sex groups of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

	Genotypes		
	GG **n (%)**	**GT** **n (%)**	**TT** **n (%)**
Male cases (n=36)	20(57.1%)	8(50.0%)	8(42.1%)
Female cases (n=34)	15(42.9%)	8(50.0%)	11(57.9%)
P		**0.568**	

Table (3): Comparison between consanguinity of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

Consanguinity	**G894T Genotypes**		
	GG **n (%)**	**GT** **n (%)**	**TT** **n (%)**
Positive cases (n=15)	8(22.9%)	3(18.8%)	4(21.1%)
Negative cases (n=55)	27(77.1%)	13(81.2%)	15(78.9%)
P		**0.945**	

Table (4): Comparison between Family history of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

Family history	**G 894T Genotypes**		
	GG **n (%)**	**GT** **n (%)**	**TT** **n (%)**
Positive cases (n=14)	7(20.0%)	4(25.0%)	3(15.8%)
Negative cases (n=56)	28(80.0%)	12(75.0%)	16(84.2%)
P		**0.794**	

Table (5): Comparison between smokers of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

Smoker	**Genotypes**		
	GG **n (%)**	**GT** **n (%)**	**TT** **n (%)**
Positive cases (n=19)	12(34.3%)	3(18.8%)	4(21.1%)
Negative cases (n=51)	23(65.7%)	13(81.2%)	15(78.9%)
P		**0.401**	

Table (6): Comparison between cholesterol and triglyceride Of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

	Cholesterol Mean ± SD	TG Mean ± SD	*P*
Genotypes			
GG	189.71±61.15	175.82±103.42	
GT	181.37±70.51	219.50±186.09	>0.05
TT	202.36±64.10	283.78±237.42	

Table (7): Comparison between Lactate dehydrogenase of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was significant difference between the two groups.

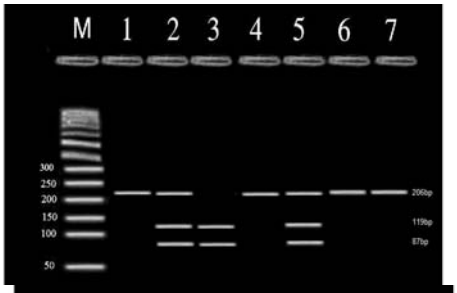
LDH Mean ± SD	GG	**Genotypes** GT	TT
< =403cases (n=42)	392.82±325.70	440.88±342.79	372.41± 318.39
>403 cases (n=28)	415.02±338.71	279.18±253.69	488.52±354.40
P		**0.007**	

Chi-square=9.7

Table (8): Comparison between liver enzyemes of CAD subjects, regarding their genotype distribution of eNOS gene polymorphisms. There was no significant difference between the two groups.

	GPT Mean ± SD	GOT Mean ± SD	P
Genotypes			
GG	11.94±8.17	18.31±11.68	
GT	7.25±4.66	11.81±7.78	>0.05
TT	10.05±8.29	14.94±10.72	

Fig (1) Enzymatic digestion of G894T polymorphism of eNOS gene . Wild type GG is found which appears at 206 bp only lanes 1,4,6 and 7 digestion of PCR product of G894T polymorphism of eNOS gene using *MboI* 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 5) (homozygous mutated genotype TT is found which has 119,87 bp fragments lanes 3) (By using DNA size marker 50 bp).



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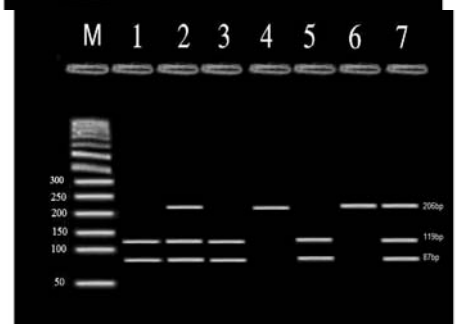

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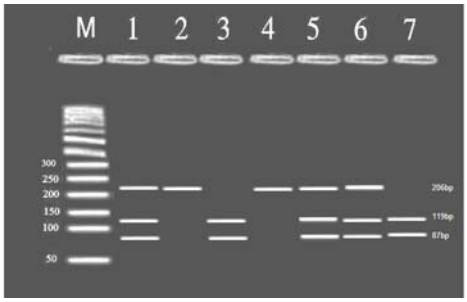

4. Discussion

Coronary artery disease is a multifactorial genes and environmental factors **(Tuomisto *et al.*, 2005)**. Environmental and genetic factors influence a person's blood in terms of fat, or lipid levels, and important risk factors for coronary artery disease (CAD).

Analyzing studied on Egyptian cases for combined genotypes, a certain pattern could be found to play a role in coronary artery disease susceptibility and/or severity.

Our study showed that there was significant difference in genotype distribution of G894T polymorphism of eNOS gene among coronary artery disease patient and control .The frequencies of the GG, GT, and TT genotypes in exon 7 for the CAD group were 50.0%, 22.9% and 27.1% respectively, and for the control group they were 67.7%, 27.4% and 4.8% respectively. There were significant differences in homozygous mutant GG and TT (P=0.039 and P=0.001**)** respectively. The frequency of the G allele was (61.4%) in CAD group and (81.5%) in the control group. So,there was significant difference in this allele frequencies (P=0.01).Similarly, the frequency of the T allele was (38.6%) in CAD group and (18.5%) in the control group so, there was significant difference in T allele frequencies (P=0.01).

We found that our result was in agreement with what was previously reported in a Japanese population,whereas G894T polymorphism was significantly correlated with coronary spasm, myocardial infarction and acute coronary syndrome ,(P =0.0085) for genotype distribution **(Hibi *et al*. (1998).** Similarlyt the G894T polymorphism was found to be a major risk factor for CAD in a UK population, (P<0.0001) for genotype distribution and (P<0.0001) for allele distribution**(Hingorani *et al*., 1999).** The G894T polymorphism of eNOS gene is significantly and independently associated with the occurrence and severity of CAD in an Italian population. It was found that (P =0.03) for genotype distribution and (P =0.05) for allele distribution **(Colombo *et al*. 2003).** With the same manner a Caucasian population recorded significant differences in genotype frequencies between the patients with CAD and the control group (P=0.003) **(Willem *et al*., 2004)**.Likewise, G894T polymorphism is significantly associated with premature CAD in a Turkish population .The patients group showed an increase in the frequency of the T allele compared to controls (p=0.0001)**(Cam *et al*., 2005). Kerkeni *et al*. (2006)** studed a Tunisian population and found significant differences in genotype distribution of G894T and allele frequencies of the patients with CAD and the control group, (P =0.035) for genotype distribution and (P =0.026) for allele distribution. In Eastern Taiwan,CAD was significantly associated with G894TT genotype , (P = 0.004) for genotype distribution and (P = 0.005) for allele distribution **Lin *et al*. (2008).** Similarly, in Maghreb population, it was found that genotype distribution of the G894T genotypes significantly differed in CAD cases and controls (p=0.025) **(Meroufell *et al*., 2009).** Recently, a Saudi researcher recorded statistically significant difference with (p <0.0001) for genotype distribution and (p= 0.002)for allele distribution.This study,firstly suggested an independent association of G894T polymorphisms of endothelial nitric oxide synthase gene with coronary artery disease **(Alkharfy *et al*., 2010).**On the same line **Dafni *et al*. (2010**) studed a Greek population and demonstrated that there were significant differences in genotype and allele frequencies between the patients with CAD and the control group (P =0.046 , for genotype distribution while P =0.019 , for allele Furthermore,studies in non-Asian populations showed a positively significant association, (p = 0.003) for the genotype distribution of G894T polymorphism of eNOS gene and (p = 0.004) for allele distribution **(Junyan *et al*., 2010)**.The same results were reported by **Syed *et al*. (2010)** in cases of South Indian population.There was, statistically, significant difference in the frequency of a specific allele/genotype between patients and their controls which may indicate a risk amounting to CAD ,(P =0.024 for genotype distribution and P =0.005 for allele distribution.

On the other hand, our results disagree with that reported in Saudi Arabia by **Yen *et al*., (2001).**They showed that the association between genotypes polymorphism and cardiovascular diseases are not consistent, (p=0.134) for genotype and (p=0.134) for allele distribution. The frequency of the T allele was 10.1% in the premature CAD group and 10.8% in the control group (p=0.134). In Taiwan, we also disagree with **George *et al*., (2008)** who showed that ,the prevalence of the Asp298 variant of eNOS was not found to be significantly and independently associated with risk of CAD (P = 0.663).Thus homozygosis for the Asp298 variant of the G 894T polymorphism in the eNOS gene was not found to be associated with risk of CAD. **Morerecent, Al-Faris *et al*. (2011)**showed that, the frequency of the GG,GT and TT genotype was not found to differ significantly in the tested cases and their controls and independently associated with the risk of CAD (P = 0.663).So, these results have not validate the association between G894T polymorphism, in the eNOS gene and increased risk of CAD. **Conclusions:** The Controversies may be

explained by the assumption that these genotypes are population specific and co-segregate with the disease genes in different forms among different ethnic groups. Based on this study, we can conclude that endothelial nitric oxide synthase (eNOS) G894T gene polymorphisms may be considered as genetic markers for coronary artery disease among Egyptian cases.

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