



Correlation of Glu298Asp eNOS Polymorphism with Serum NO Levels in Egyptian Patients with Coronary Artery Disease

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Authors' contributions

All authors contributed in the design, analysis, writing and reviewing of the manuscript. All authors read and approved the final manuscript.

Research Article

Received 23rd March 2013
Accepted 20th May 2013
Published 24th May 2013

ABSTRACT

Background: Nitric oxide (NO) is a potent vasodilator synthesized by the vascular endothelium. It has been reported that endothelial nitric oxide synthase (eNOS) Glu298Asp gene polymorphism is associated with coronary artery disease (CAD).

Methodology: In this study, we investigated Glu298Asp eNOS polymorphism and serum NO in a group of 146 age-matched male subjects; 77 patients with coronary artery disease (CAD) classified according to the severity of coronary insufficiency and 69 normal male controls.

Results: The obtained frequencies of the eNOS Glu298Asp genotypes for the CAD subjects were GG (54.5%), GT (31.20%), and TT (14.3%). The allele distributions of G and T were 70.1 and 29.9%, respectively. In the control group, the genotype frequencies were 53.6% for GG, 36.2% for GT, and 10.2% for TT, and the frequencies of the G and T alleles were 71.7% and 28.3%, respectively. There were no significant differences in genotype and allele frequencies between the CAD patients and the control group. The mean serum NO levels in CAD patients was significantly higher than that of healthy subjects ($p=0.0139$).

Conclusion: No significant association was detected when CAD severity, genotypes and NO serum levels were correlated.

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Keywords: *Endothelial nitric oxide synthase; gene polymorphism; coronary heart disease; serum nitric oxide.*

1. INTRODUCTION

Coronary artery disease(CAD) is an important health public problem in many development nations [1], and is a major cause of mortality in Egypt [2]. The vascular endothelium plays a vital role in regulating local blood flow and homeostasis. Many studies have shown that endothelial dysfunction predisposes patients of all ages to a variety of cardiac and vascular disease states [3]. Nitric oxide (NO) formation is of particular importance for normal endothelial function. It is produced by oxidation of L-arginine to L-citrulline by the endothelial nitric oxide synthase (eNOS) [4]. Once NO is produced, it diffuses from the endothelium to the vascular smooth muscle cells, where it increases the concentration of cGMP, leading to vascular relaxation [5]. In addition, NO has vasoprotective effects by scavenging superoxide radicals and suppressing platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation [6], and also limits the oxidation of atherogenic low-density lipoproteins [7].

The functional disturbance of the NO synthesis and degradation is considered an early [8] and reversible [9] event in the pathogenesis of numerous cardiovascular diseases. NO is regulated at the level of its synthesis by the gene encoding eNOS. Several polymorphisms in the eNOS gene were reported to be related to the pathogenesis of coronary artery disease[10]. Among these is the G984T in exon 7, which results in an amino acid sequence change from Glu 298 to Asp. Tesauro et al. [11] suggested that eNOS Glu298Asp polymorphism might display low eNOS activity leading to decreased generation of NO and increased superoxide level.

Recently, several studies have evaluated the association of eNOS Glu298Asp polymorphism and the risk of CAD. However, only a few studies were conducted in Egypt, using a limited number of subjects [6,12]. In this study, we used a group of CAD Egyptians to study the relationship between eNOS Glu298Asp polymorphism, the severity of coronary insufficiency, and the serum levels of NO aiming to shed more light on the genetic susceptibility to CVD in Egyptians.

2. SUBJECTS AND METHODS

2.1 Study Population

Seventy seven CAD male Egyptian subjects were recruited in this study with a 35-50 years age range. They were admitted to in- and out-patient clinics of the National Heart Institute (NHI), Imbaba, Cairo, Egypt and angiographically diagnosed. The patients were further classified according to the severity of coronary insufficiency and type of management into four groups; (1) patients under conservative medical treatment (Med, n=9), (2) patients directed for percutaneous coronary interventions (PCI, n= 31), (3) patients advised to have a coronary artery bypass graft operation (CABG, n=25), and (4) patients suffering from acute myocardial infarction (AMI, n= 12). The controls were drawn from normal, healthy, age and sex matched volunteers attending the blood bank of 57357 Children Cancer Hospital, Cairo, Egypt. They were included if they had no clinical or diagnostic evidence for CAD, diabetes mellitus and having controlled blood pressure below 140/90 mmHg. All recruited subjects gave written informed consents that complied with the principles of the Helsinki declaration.

The study protocol was approved by the local ethics committee at the German University in Cairo.

2.2 Genotyping Assay

Genomic DNA was prepared from whole blood using a QIAamp DNA blood minikit (Qiagen) according to the manufacturer's instructions. Genotypes for the Glu298Asp polymorphism were determined by PCR-restriction fragment length polymorphism (PCR-RELP) analysis using specific oligonucleotide primers: forward (sense) 5'-TCCCTGAGGAGGGCATGAGGCT-3' and reverse (antisense) 5'-TGAGGGTCACACAGGTTCCCT-3'. PCR products were digested by *BanII*, and separated by electrophoresis using agarose gel (2%) and visualized by ethidium bromide staining. The wild type allele G has *BanII* cutting site producing smaller fragments (137 and 320 bp). In case of a G to T substitution, this *BanII* recognition site is lost [13].

2.3 Nitric Oxide (NO) Assay

Serum NO levels were measured as total nitrite concentration using Griess reagent (2% w/v sulphanilamide in 5% HCL and 0.1% naphylethanolamine in H₂O) [14], after conversion of nitrate to nitrite by vanadium (III) chloride (VCL₃). Calibration curves were plotted for potassium nitrate in distilled H₂O (0-100 µmol/L). Absorbance was measured spectrophotometrically at 540 nm.

2.4 Statistical Analysis

Deviation from the Hardy-Weinberg equilibrium was assessed using χ^2 test with one degree of freedom. Values were expressed as means \pm standard error of the mean (SEM). The significance of difference was analyzed by using Mann Whitney test U-test or Kruskal- Wallis test. Allele frequencies were calculated from the genotypes of all subjects by the gene counting method and compared using the χ^2 analysis. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were also determined. Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Genotype and Allele Frequencies of eNOS Glu298Asp Gene Variants in Patients with CAD and Control Subjects

A total of 146 male subjects were enrolled in this study; 77 patients with CAD and 69 healthy controls. The genotype distribution and the relative allele frequency of the eNOS Glu298Asp gene polymorphism in CAD patients and control subjects are shown in Table 1. The genotype frequencies of the Glu298Asp polymorphism did not significantly differ from those predicted under conditions of Hardy-Weinberg equilibrium ($P > 0.05$).

The frequency of the TT genotype for the G894T polymorphism was 14.3% in the CAD patients and 10.2% in the control group. No significant difference was found between the two groups ($P > 0.05$). The allele frequencies of the G to T transition in the healthy and CAD groups were 28.3% and 29.9%, respectively (Table 1) which showed no significant difference as well. The combination of the GT and GG genotypes with respect to the TT genotype was not a risk factor for CAD (OR=1.05; 95% CI; 0.56 - 1.9; $P = 1.0$).

Table 1. Genotype and allelic frequencies in controls and CAD patients of Glu298Asp polymorphism

Group	Total no	Genotypes (n %)			Alleles (n %)		Odds ratio (OR) CI (95%)	<i>P</i>
		GG (Glu/Glu)	GT (Glu/Asp)	TT (Asp/Asp)	G (Glu)	T (Asp)		
Control	69	37 (53.6%)	25 (36.2%)	7 (10.2%)	99 (71.7%)	39 (28.3%)	OR= 1.05	<i>P</i> =1.0
CAD patients	77	42 (54.5%)	24 (31.2%)	11 (14.3%)	108 (70.1%)	46 (29.9%)	CI = (0.56 - 1.9)	

These findings are similar to those previously described by other investigators from different populations. In a sample of Turkish population, no significant increase was found in the GG, GT and TT genotypes of patients with CAD as compared to healthy controls [15]. In Japanese and Korean patients, no association was also found between the missense Glu298Asp variant and CAD [16,17]. Similar conclusion was derived from Chilean study [18]. However, findings of two studies from the UK were contradictory. While one of these studies showed that the missense Glu298Asp variant was a major risk factor in white population from the UK [19], the other study informed that this variant was not associated with CAD [20]. A recent meta-analysis of 14 studies, involving 6036 subjects with CAD and 6106 controls, showed that the exon 7 polymorphism of the eNOS gene contribute to atherosclerosis susceptibility, presumably by effects on endothelial NO availability [21].

3.2 Comparative Analysis of Glu298Asp Genotype among CAD Groups and Control Subjects

The 77 CAD patients were classified into 4 groups according to the severity of coronary insufficiency as verified by coronary angiography. The genotype frequency of the Glu298Asp of the eNOS gene in different CAD groups and control subjects are shown in Fig. 1. The genotype frequencies in the PCI and CABG groups showed no significant differences from their respective controls. None of the medically treated group subjects (Med) carried the TT genotype. The frequency of the TT homozygotes in patients with AMI was significantly higher than that of control, PCI and CABG groups. This goes positively with the results of Shimasaki et al. [13], who reported an association of the Glu298Asp gene with the risk of MI. They found that T allele carriers had a 1.7-fold increased risk of MI.

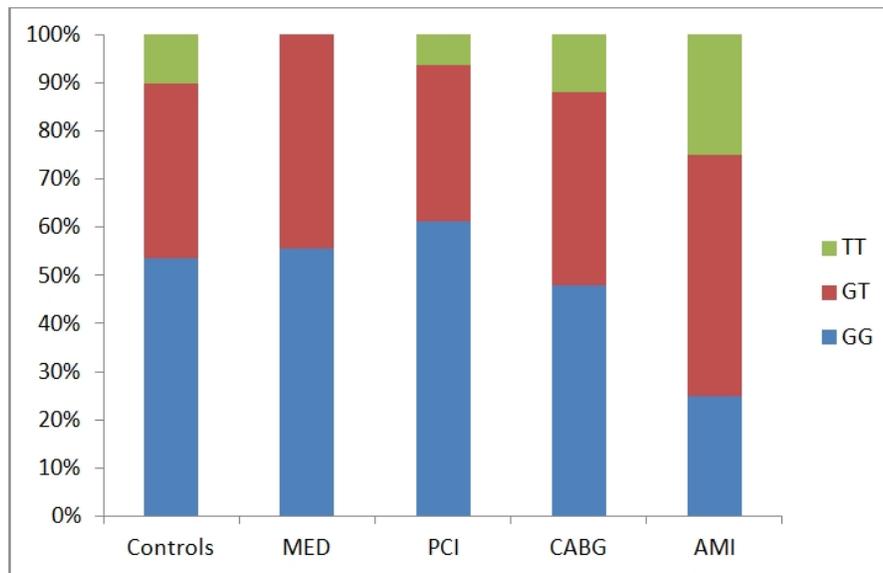


Fig. 1. Distribution of Glu298Asp eNOS genotypes among controls and coronary artery disease (CAD) classes

The CAD patients were classified into 4 subclasses; (1) patients under conservative medical treatment (Med, n= 9), (2) patients directed for percutaneous coronary interventions (PCI, n = 31), (3) patients advised to have a coronary artery bypass graft operation (CABG, n =25), (4) patients suffering from acute myocardial infarction (AMI, n = 12) and control subjects (n=69).

Similarly, Colombo et al. [21] reported that the risk of developing CAD was about threefold higher for T homozygotes than in person with a G allele in the eNOS gene. Similarly, in a previous study done on a group of Egyptian CAD patients, Motawi et al [12], found a strong association between Glu298Asp eNOS polymorphism and the risk of ischemic heart disease. This increased risk was confined to individuals homozygous for the Asp. In another study a significant increase of the T homozygotes was detected among individuals with recent acute MI when compared to healthy controls and the odds ratio for acute MI among T homozygotes was 2.5 times that of the G homozygotes [22].

In contrast to the previous results, Nassar et al. [23] did not find any association between the G894T polymorphism and CAD incidence in Canadian subjects with a history of MI or angina pectoris plus angiographically documented CAD. Aras et al. [24] reported that T homozygote of the Glu298Asp eNOS polymorphism has no significant effect on the risk and extent of CAD in the Turkish population. In harmony, a case control study on 531 patients with MI and 610 control subjects showed no association of eNOS Glu298Asp polymorphism with MI [25].

These contradictory results may be explained, at least in part, by the ethnic variations [26,27], the gene-gene and gene-environment interactions [28], and the great variability in sample sizes investigated in these studies [29].

3.3 NO Serum Levels in CAD Patients and Control Subjects

Mean serum NO level in CAD patients was significantly higher by 20% than that of control subjects (36.1 μ M and 30.0 μ M, respectively) (Fig. 2).

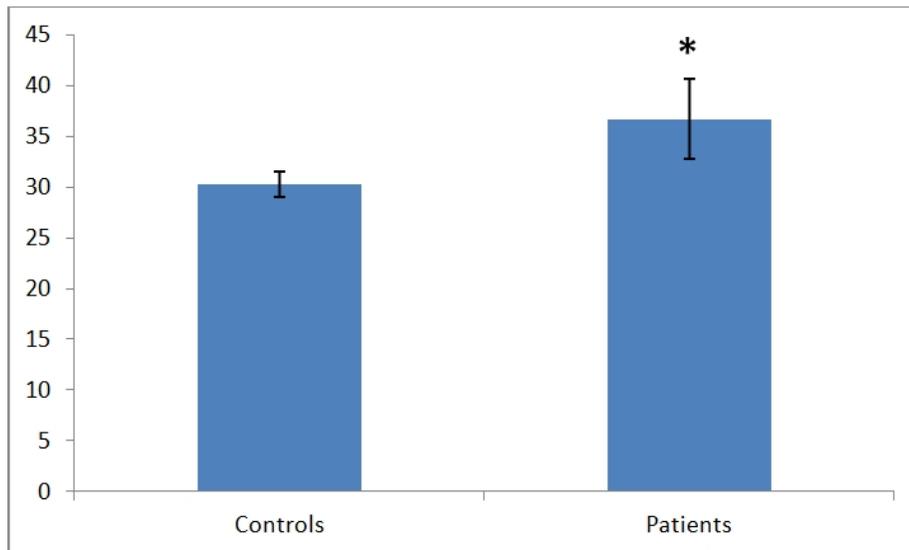


Fig. 2. Serum levels of NO in controls and CAD group.
NO serum levels showed a significant difference at $P = 0.014$

In fasted individuals, about 90% of the circulating NO_2^- is derived from the L-arginine nitric oxide pathway, and NO_2^- therefore is a valid indicator of NO production [30]. The patients in our study had abstained from cigarette smoking, which itself was reported to contain NO,

and food for at least 12 h and drinking for at least 8 h before the blood samples were collected; therefore, smoking and dietary sources are more likely to be excluded as a possible source of the observed increase in serum NO levels. Accordingly, the increased level of serum NO in CAD patients is suggestive of cumulative increase in NO synthesis. Few studies reported similar increases in plasma NO concentration in CAD patients. Yoon et al. [17] found that median plasma NOx was significantly higher ($P < 0.001$) in CAD patients ($95.9 \mu\text{mol/L}$) than in controls ($73.8 \mu\text{mol/L}$). This finding suggests the compensatory increase in plasma NO in response to increased superoxide anion concentration [29] and shear stress in blood vessels [30] in CAD patients. Another possible explanation is the increased production of NO level by the inducible nitric oxide synthase (iNOS) that becomes activated in CAD [31,32]. However, it remains to be known whether the increase in plasma NO is caused by or is a result of the impairment of endothelial function.

3.4 Association of eNOS Glu298Asp Genotypes with Mean Serum NO Levels among CAD Group and Control Subjects

Serum NO values were correlated with genotypes of Glu298Asp of the eNOS gene in CAD patients and controls. In the control group, mean serum NO concentrations were 28.6, 31.0 and 33.8 μM for GG, GT and TT, respectively. In CAD patients, mean levels were 42.5, 36.4 and 41.9 μM in the GG, GT and TT, respectively (Table 2). There was no significant difference among the three Glu298Asp genotypes in both controls and patients ($P > 0.05$).

Table 2. Comparison of serum NO levels between the genotypes of the eNOS Glu298Asp gene polymorphism

Group	Total No	GG		GT		TT	
		n	NO(μM)	n	NO(μM)	n	NO(μM)
Controls	69	37	28.6 ± 2	25	31.0 ± 2.56	7	33.8 ± 5.68
CAD patients	77	42	42.5 ± 2.56	24	36.4 ± 2.64	11	41.9 ± 6.25

Only few data were published about the correlation between genetic polymorphism in eNOS gene and serum NO levels. In the present study, a lack of association between eNOS genotypes and serum NO level in CAD patients and controls was evident. Our results are consistent with the studies done on Turkish population, which showed no significant difference in serum NO values among the three Glu298Asp genotypes in both controls and patients ($P = 0.231$, $P = 0.243$, respectively) [15]. Yoon et al. [17] showed that only in the control group, plasma NO was significantly dependent on the genotypes of the E298D polymorphism; this relationship was not observed in CAD patients.

4. CONCLUSION

In summary, our data further support the reported increase in plasma NO in CAD patients, yet no enough evidence exists on the potential contribution of Glu298Asp polymorphism in the eNOS gene in the regulation of plasma NO levels.

CONSENT

All authors declare that 'written informed consent was obtained from the patient for publication of this study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients did not receive any treatments either physically or medically.

ACKNOWLEDGEMENTS

This study was supported by the Science and Technology Development Fund (STDF) grant No. 2951.

COMPETING INTERESTS

Authors have declared that no competing interests or potential conflicts of interest exist.

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