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The Glu-298→Asp (894G→T) mutation at exon 7 of the endothelial nitric oxide synthase gene and coronary artery disease

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Abstract We examined associations between the endothelial nitric oxide synthase (eNOS) gene Glu-298→Asp (894G→T) mutation and the occurrence and severity of angiographically defined coronary artery disease (CAD). eNOS mediates basal vascular wall nitric oxide production, and altered nitric oxide production has been implicated in atherosclerosis. The newly identified eNOS Glu-298→Asp mutation in exon 7 is common and likely to be functional. It was found to be associated with myocardial infarction (MI) in Japanese but not in whites. We genotyped 763 white Australians undergoing coronary angiography for the eNOS Glu-298→Asp mutation. The frequencies of the eNOS GG, TG and TT genotypes were 47.8%, 41.2% and 11.0% in men and 45.2%, 41.1% and 13.7% in women with CAD, and were not significantly different from those without CAD (43.2%, 40.7% and 16.0%, P=0.423 in men; 40.2%, 48.1% and 11.7%, P=0.582 in women). The mutation was also not associated with MI (P=0.469 in males; P=0.389 in females) or with the number of significantly stenosed vessels (P=0.954; P=0.734). The "T" allele frequency (32.5%) was much greater than that reported for the Japanese population (7.8% in controls and 10.0% in MI patients). In conclusion, the eNOS Glu-298→Asp mutation is common, occurring with an allele frequency of 32.5%, but is not associated with either the occurrence or severity of CAD in the Australian population or with other established coronary risk factors assessed in our study. The mutation is significantly more frequent in the Australian than in the Japanese.

Key words Endothelial nitric oxide synthase · Mutation · Coronary artery disease

Abbreviations *CAD* Coronary artery disease · *eNOS* Endothelial nitric oxide synthase · *MI* Myocardial infarction · *PCR* Polymerase chain reaction

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Introduction

Nitric oxide plays an important role in physiological regulation of vascular tone and blood pressure [1]. It is an intracellular signalling molecule which activates soluble guanylate cyclase to produce cGMP [2]. Alterations in nitric oxide production and/or bioavailability in vasculature have been implicated in ageing, cigarette smoking,

hypercholesterolaemia, and in some disease conditions including hypertension, diabetes and atherosclerosis [3, 4, 5, 6, 7, 8]. Endothelial nitric oxide synthase (eNOS, or NOS3) is the key enzyme responsible for basal vascular wall production of nitric oxide [7, 8].

The gene encoding eNOS is located on chromosome 7q35–36 and comprises 26 exons [9]. We have reported that a 27-bp repeat polymorphism at intron 4 of the eNOS gene predicts smoking-dependent risk of coronary artery disease (CAD) [10] and shown that it is associated with altered plasma nitric oxide levels [11]. Recently it was reported that a Glu-298→Asp mutation at exon 7 of the eNOS gene is associated with myocardial infarction (MI) [12, 13], coronary spasm [14] and essential hypertension in Japanese population [15]. Whilst Hibi et al. [13] observed a significant relationship between the mutation and a history of MI in the Japanese population, they also showed no association between the mutation and the severity of coronary stenosis. However, Liyou et al. [16] reported no association between the mutation and CAD in whites. Furthermore, Lacolley and colleagues [17] demonstrated that the mutation is not related to aortic stiffness in hypertensive and normotensive subjects of European ancestry, and, in contrast, that the frequency of wild-type Glu-298 homozygotes is higher in hypertensive patients.

Because the Glu-298→Asp mutation is in exon and results in an amino acid change, it could have functional effects. In view of the contradictory findings, we explored relationships between the eNOS Glu-298→Asp mutation and the occurrence and severity of CAD in well-characterized white Australian patients undergoing coronary angiography. We compared the genotype distributions of the mutation in patients with and without CAD, and in patients with different numbers of significantly stenosed (>50% luminal obstruction) major epicardial coronary arteries.

Patients and methods

The patient population

We studied 763 whites (562 men, 201 women) aged 65 years or younger, consecutively referred to the Eastern Heart Clinic at Prince of Wales Hospital for coronary angiography. The severity of CAD was determined by the number of significantly stenosed coronary arteries. Each angiogram was classified as revealing either normal coronary arteries or having no coronary lesion with more than 50% luminal stenosis or as having one, two, or three major epicardial coronary arteries with more than 50% luminal obstructions. Written consent was obtained from every patient. The study design was approved by the Ethics Committee of the University of New South Wales.

We obtained each patient's medical history using a questionnaire with standardized choices of answers to be ticked during the interview, and DNA samples were collected for each patient as described previously [10].

Determination of the eNOS exon 7 Glu-298→Asp (894G→T) mutation

The polymerase chain reaction (PCR) was used to detect the Glu-298→Asp mutation at exon 7 of the eNOS gene with the forward and reverse primers as 5'-AAGGCAGGAGACAGTGGATG-3' and 5'-CAGTCAATCCCTTTGGTGCT-3'. The amplification was performed in 25-µl volumes containing 100 ng DNA, 20 pmol of each primer, 2.1 mmol/l $MgCI_2$, 50 mmol/l KCI, 25 mmol/l dNTP, 5 mmol/l Tris-HCI (pH 8.3) and 1 U Taq polymerase. Samples were subjected to denaturing at 94°C for 5 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1.5 min and a final extension at 72°C for 5 min. The 246-bp PCR products were digested with a *Dpn*II restriction enzyme (recognition site: 5'...↓GATC...3') and run in 8% polyacrylamide gel for 1 h and silver-stained. This resulted in two fragments (158 bp and 88 bp) when the restriction site created by the 894G→T transition (5'-GAG/TC-3') was present (Fig. 1). The genotypes were identified as GG, TG and TT.

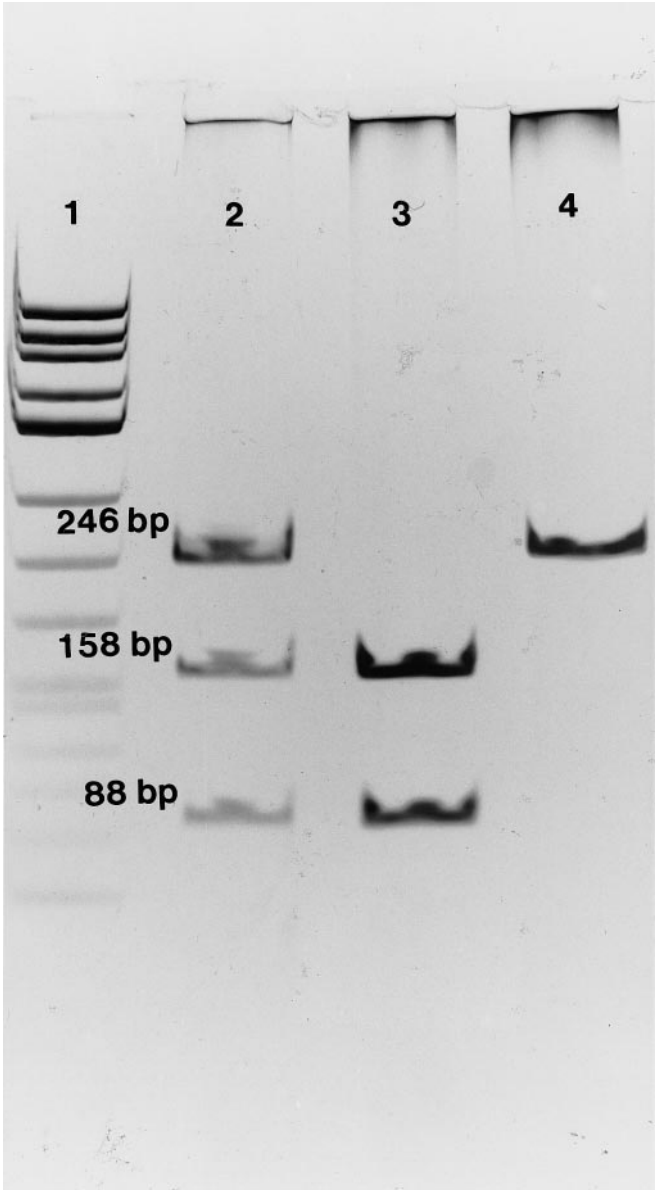

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis gel shows the PCR products from the amplification of the mutation region of the eNOS gene. The mutation creates a *Dpn*II recognition site which digests the 246-bp fragment into 158- and 88-bp fragments. *Lane 1* a molecular size marker (Promega, ϕx174 DNA/*Hin*fI); *lane 2* a TG heterozygote; *lane 3* a rare TT homozygote; *lane 4* a common GG homozygote

Statistical analysis

A computer package of SPSS Advanced Statistics 8.0 for PC Windows 95 was used to analyse the data. Hardy-Weinberg equilibrium for the eNOS Glu-298→Asp genotype distribution was assessed as before [10]. We used a one-way analysis of variance to estimate relationships between the eNOS genotypes and quantitative variables. A contingency table χ^2 analysis was employed to estimate the contribution of the mutation to the presence and severity of CAD, and to evaluate relationships between the genotypes and other medical conditions including MI, angina pectoris, family history of CAD, diabetes mellitus and hypertension. To assess the independent effect of the eNOS Glu-298→Asp mutation on CAD, other established risk factors for CAD were entered as covariates in a stepwise logistic regression model. The statistical significance was defined as $P<0.05$ and two-tailed P values are reported.

Results

The frequencies of the eNOS Glu-298→Asp (894G→T) genotypes were 46.8%, 41.6% and 11.7% for GG, TG and TT, respectively, and the mutant "T" allele frequency was 32.5%. The genotype distribution was in Hardy-Weinberg equilibrium ($\chi^2=2.076$, $P>0.05$) and was not different between men and women ($\chi^2=0.837$, $P=0.658$).

The age and frequencies for the eNOS GG, TG and TT genotypes and the allele frequencies in patients with and without angiographically defined CAD are shown in Table 1. There were no significant associations between the eNOS genotypes and the presence of CAD ($\chi^2=1.722$, $P=0.423$ in men; $\chi^2=1.084$, $P=0.582$ in women). No excess of rare TT homozygotes or "T" allele was observed in patients with CAD compared to those without. Both male and female patients with CAD tended to be older, although the difference was statistically significant only in men (Table 1). In a stepwise logistic regression analysis in which CAD was entered as the dependent variable and age, body mass index, waist/hip ratio, smoking status and lifetime smoking dose, lipid levels (total cholesterol, triglycerides, low-density lipoprotein cholesterol, total cholesterol/high-density lipoprotein cholesterol, lipoprotein (a), apolipoproteins AI and B), diabetes and/or hypertension and family history of CAD were entered as independent variables, the eNOS genotypes were still not predictive of the occurrence of CAD after controlling for these confounding risk factors ($P=0.783$ in men; $P=0.992$ in women), nor was the mutation associated with any of the above risk factors.

As shown in Table 2, the mutant Asp-298 homozygotes in patients with a past history of MI were not more prevalent than in patients without ($\chi^2=1.513$, $P=0.469$ in men; $\chi^2=1.890$, $P=0.389$ in women). Similarly, the eNOS genotypes were not associated with the presence of angina pectoris ($P=0.087$; $P=0.943$). There was no consistent relationship between the frequency of the Asp-298 homozygotes or of the "T" allele frequency and the number of significantly stenosed vessels ($\chi^2=1.576$, $P=0.954$ in men; $\chi^2=3.574$, $P=0.734$ in women). The eNOS genotypes were also not associated with family history of CAD ($P=0.417$; $P=0.133$), diabetes ($P=0.586$; $P=0.953$) or hypertension ($P=0.844$; $P=0.256$). Furthermore, when the analysis was conducted in smokers and

Table 1 Distribution of the eNOS Glu-298→Asp (894G→T) mutation in patients with and without angiographically defined CAD (percentages in brackets)

	Men		Women	
	With CAD	Without CAD	With CAD	Without CAD
Age (years)	56.4±7.2	52.1±9.3*	58.4±7.3	56.3±7.7
eNOS genotypes				
TT	53 (11.0%)	13 (16.0%)	17 (13.7%)	9 (11.7%)
TG	198 (41.2%)	33 (40.7%)	51 (41.1%)	37 (48.1%)
GG	230 (47.8%)	35 (43.2)	56 (45.2%)	31 (40.2%)
Alleles				
T	0.32	0.36	0.34	0.36
G	0.68	0.64	0.66	0.64

*$P<0.01$ by Student's t test. The genotype distribution among patients with CAD did not differ from that in those without (men: $\chi^2=1.722$, $P=0.423$; women: $\chi^2=1.084$, $P=0.582$)

Table 2 Distribution of the eNOS Glu-298→Asp (894G→T) mutation in patients with and without a past history of MI (percentages in brackets)

	Men		Women	
	With MI	Without MI	With MI	Without MI
Age (years)	56.1±7.8	55.5±7.6	57.3±7.5	57.9±7.5
eNOS genotypes				
TT	28 (12.0%)	36 (11.0%)	7 (9.7%)	19 (14.7%)
TG	102 (43.6%)	129 (39.3%)	35 (48.6%)	53 (41.1%)
GG	104 (44.4%)	163 (49.7%)	30 (41.7%)	57 (44.2%)
Alleles				
T	0.34	0.31	0.34	0.35
G	0.66	0.69	0.66	0.65

The genotype distribution among patients with a MI history did not differ from that in those without (men: $\chi^2=1.513$, $P=0.469$; women: $\chi^2=1.890$, $P=0.389$)

non-smokers separately, the eNOS Glu-298→Asp mutation was not associated with either the presence or severity of CAD, MI, angina pectoris, diabetes, hypertension and family history of CAD.

Discussion

Our study in white Australians shows no association between the eNOS exon 7 Glu-298→Asp (894G→T) mutation and either the presence or the severity of CAD in men or women. This is supported by the findings of Hibi et al. [13] in Japanese and Liyou et al. [16] in whites. We also report no relationship between the eNOS mutation and a history of MI or the presence of angina pectoris or with family history of CAD, diabetes and hypertension. This is consistent with our previous report that the mutation is not related to macro- or microvascular complications in Australian middle-aged type 2 diabetic patients [18]. Furthermore, the wild-type GG genotypes tended to be more frequent in patients with CAD than in those without (Table 1). Similar observations have been reported by Lacolley et al. [17] among Europeans and are supported by the findings of Markus et al. [19] that the mutation was not associated with ischemic cerebrovascular disease in white subjects.

The eNOS GG, TG and TT frequencies in the 763 white patients undergoing coronary angiography were 46.8%, 41.6% and 11.7% and not statistically different from the 51.9%, 40.6% and 7.5% that we reported in 574 Australian type 2 diabetic patients [18]. In the European hypertensives recruited in the study of Lacolley et al. [17] the GG, TG and TT frequencies were 45%, 40% and 15% and were also not significantly different from those of ours. However, there was a much lower frequency of the mutant allele in the Japanese subjects. Shimasaki et al. [12] reported the frequencies 78.9% (225/285), 20.7% (59/285) and 0.4% (1/285) for GG, TG and TT genotypes in Japanese MI patients compared to 86.7% (526/607), 13.2% (80/607) and 0.2% (1/607), respectively, in controls. These figures are similar to those of another Japanese population reported by Hibi et al. [13] (83.6%, 14.2% and 2.2% for GG, TG and TT genotypes in MI and 82.6%, 17.4% and 0.0% respectively in controls). To sum these two populations together, the "T" allele frequency in Japanese population was 10.0% in MI and 7.8% in controls which are similar to the findings of Miyamoto et al. [15] (the T allele in Japanese hypertensives: 10.3%, controls: 5.0%). Therefore The "T" allele frequency in the Australian population (32.5%) is much higher than that in the Japanese (7.8%–10.0%). This significant difference in the mutation frequency between whites and Japanese suggests that the genetic marker is population specific, which could also explain the inconsistent findings between populations.

In conclusion, the eNOS exon 7 Glu-298→Asp mutation occurs commonly in white Australians, with an allele frequency of 32.5%. It is not associated with CAD occurrence or severity documented angiographically or with the established coronary risk factors assessed in the present study. The mutation is unlikely to be a functional one but rather a polymorphic marker, which is significantly more frequent in the Australian than in the Japanese population.

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