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ORIGINAL PAPER

Methylentetrahydrofolate Reductase and Nitric Oxide Synthase Polymorphism in Patients with Atherosclerosis and Diabetes

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Abstract The development of the atherosclerosis is based on multifactorial causes. In addition to the traditional risk factors, gene polymorphisms can play a role in the disease. Therefore in this study we investigated whether the eNOS and MTHFR gene polymorphisms is associated with myocardial infarction and stroke in patients with or without diabetes. We have identified polymorphisms in the *NOS 3* gene and one of these polymorphisms, $Glu^{298}\rightarrow Asp$, was found to be a major risk factor for carotid artery disease and myocardial infarction. Our results indicate that the *MTHFR* G677T allele is significantly associated with MI. *MTHFR* 677 G/T genotyping may be of clinical importance as a prognostic and therapeutic marker, although further studies are needed to substantiate this hypothesis.

Keywords Nitric oxide synthase · Methylentetrahydrofolate reductase · Polymorphism · Atherosclerosis · Cardiovascular Risk

Abbrevations

MTHFR	methylentetrahydrofolate reductase
eNOS	endothelial nitric oxide synthetase
CHD	coronary heart disease
CVD	cardiovascular disease
CAD	carotis artery disease
MI	myocardial infarction

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Introduction

Coronary heart disease (CHD) is the leading cause of death in industrialized nations. Atherosclerosis is the consequence of complex interactions between genetic and environmental factors and is the primary cause of heart disease and stroke in westernized societies [1].

Traditional risk factors allow the prediction only about 50% of the absolute risk of a cardiovascular event in individual patients. A number of chronic diseases, including cardiovascular disease (CVD), appear to have a multifactorial genetic risk component [2]. Established risk factors for cardiovascular disease in the general population include age, gender, diabetes mellitus, obesity, high serum cholesterol levels, and hypertension, as well as cigarette smoking and physical inactivity. These factors, however, do not explain all premature CVD cases. A number of these established factors have genetic components, and as yet unknown risk factors may be primarily genetic [3, 4]. In addition, recent evidence indicates that genetic factors influence patient responsiveness to therapeutic intervention.

The initiating event of atherosclerosis is the endothelial dysfunction followed by smooth muscle proliferation. Exposure of the endothelial surface to a variety of risk factors such a hypertension, hyperglycemia, oxidized low density hipoprotein (LDL), infection and other inflammatory compound result in endothelial injury [5].

It is now accepted that the decreased bioavailability of endothelial nitric oxide (NO) plays a crucial role in the development and progression of atherosclerosis [6].

Initial studies suggest that increasing endothelial nitric oxide synthetase (eNOS) gene expression would improve endothelial NO release [7].

The principla hypothesis is that homozygosity to eNOS Asp298 would be associated with an increased risk to ischemic heart disease. Several studies have evaluated the association of eNOS polymorphism (Glu 298 Asp) and the risk of atherosclerosis, but data of case-control studies are conflicting [8–10].

Polymorphisms of methylentetrahydrofolate reductase (MTHFR) has also been supposed to play a role in the development of atherosclerosis [11].

Some studies [12, 13] have concluded that individuals with the MTHFR 677 TT genotype have significantly elevated plasma homocysteine levels and could have a significantly higher risk of coronary heart disease, particularly in the setting of low folate levels. Despite the well-established association between hyperhomocysteinemia and cardiovascular disease, the role of this polymorphism as a genetic risk factor of coronary artery disease is still controversial [14]. The prognostic impact of the MTHFR genotype in the clinical outcome of cardiovascular patients remains uncertain.

Recently, atherosclerotic cardiovascular diseases (CVD) are believed to be multifactorial and results from many genes working alone or in combination with modifier gene and environmental factors.

The identification and characterization of these genes would enhance the prediction of the risk of atherosclerosis and improve prevention and treatment of the disease.

Patients with type 2 diabetes are featured by substantially increased risk of atherogenesis.

Therefore in this study we investigated whether the eNOS and MTHFR gene polymorphisms is associated with myocardial infarction in patients with or without diabetes.

Our finding supports the proposal that genetic variation in the eNOS gene (Asp 298) contributes to the susceptibility of atherosclerosis.

Matherials and Methods

Subject Populations

Our studies were based on the investigations: **1**. 118 patients with acute myocardial infarction (AMI) **2**. 101 patients with diabetes and AMI, **3**. 158 patients with diabetes and without AMI, **4**. 384 control persons without diabetes and AMI.

All the patients had undergone vascular reconstructive surgeries in the Cardiovascular Department of the Semmelweis University, Budapest. Their complete medical history and laboratory tests were recorded.

All the 384 control subjects visited the outpatient clinics of the National Institute of Oncology for an annual health checkup. They had no history of ischemic or hemorrhagic stroke or other cerebral diseases, coronary heart disease, or other atherosclerotic diseases, or other thrombotic, embolic, or hemorrhagic disorders.

Measurements of Serum Markers HDL Cholesterol, LDL Cholesterol, Triglicerid

Blood samplex drawn from the subjects were collected in EDTA-containing tubes. Total cholesterol and HDL cholesterol levels triglycerid were measured by standard laboratory techniques.

Isolation of DNA Samples

DNA was extrated from the buffy coat fraction of centrifuged blood using the QIAmp Blood Kit (Qiagen).

SNP Analysis of eNOS G298T and MTHFR C677T

The following primers and probes were designed by LightCycler probe design software 2.0

eNOS*:* sense primer: 5′-CAGCTCTGCATTCAGCAC-3′; antisense primer: 5′-CATCCCAC-CCAGTCAATC-3′; sensor: 5′-LCRed640-GTTCTGGGGG**C**TCATCTGGG-PH-3′(designed for the wild type allele); anchor: 5′-CAGCTCGGGGGGCAGAAGGAA-FL-3′; ***MTHFR***: sense primer: 5′-AGGCCAGCCTCTCCTGACTG-3′; antisense primer: 5′-AGGACGGTGCGGTGAGAGTG-3′; sensor: 5′-CGGGAGCCGATTTCATCA-FL-3′; anchor: 5′-LCRed640-CGCAGCTTTTCTTTGAGGCTGACA-PH.

Amplification of 50 ng DNA template with primers (0.5 μM each) and fluorescent probes (0.2 μM each) were performed in LightCycler instrument (Roche Diagnostics GmbH) using LightCycler FastStart DNA Master HybProbe

Table 1 Clinical characteristics of patients without diabetes

	With MI	Without MI
Sex M/F 183/118	73/45	110/73
Fasting plasma glucose	5,72±1,67	5,77±1,69
Total cholesterol	5,56±1,12	5,2±0,9
Triglyceride	2,25±1,29	1,72±0,66
HDL cholesterol	1,17±0,4	1,57±0,55
LDL cholesterol	4,2±1,28	2,78±1,21

Data are means ± S.D. MI = myocardial infarction

The lipid disorder of atherosclerotic patient with or without MI are significantly different

(p<0,005)

Table 2 Clinical characteristics of patients with type 2 diabetes

	With MI	Without MI
Sex M/F 170/89	71/30	99/59
Fasting plasma glucose	10,38±3,96	10,05±3,65
Total cholesterol	5,8±2	5,6±1,29
Triglyceride	2,34±1,2	2,29±1,62
HDL cholesterol	1,21±0,29	1,25±0,34
LDL cholesterol	4,3±1,33	4,1±0,8

The lipid disorders of diabetic patients are similar in patient with or without myocardial infarctus (MI)
($p<0,01$)

mix (Roche Diagnostics GmbH). The $MgCl_2$ concentration was 2 mM. The cycling conditions of *eNOS* fragment were: 95°C 10 min followed by 45 cycles of 95°C 10 sec, 65°C 5 sec, 72°C 5 sec. The thermal profile of the ***MTHFR PCR*** was: 95°C 10 sec followed by 45 cycles of 95°C 3 sec, 52°C 5 sec and 72°C 13 sec. For the analysis of the melting curves at the end of the PCR, temperature was raised to 95°C, lowered to 45°C, then slowly raised to 85°C whilst monitoring the fluorescence intensity. The negative first derivative of the melting curves created by the software (LC vs.3.5) made possible the distinction of the genotypes.

Sequencing

Sequencing analyses were performed with the PCR products using the primers described in the SNP analysis. The sequencing reactions were carried out by BigDye terminator cycle sequencing kit v.3.1. (Applied Biosystems, Foster City, CA, USA) and the reaction products run in ABI-PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Descriptive data are presental as mean ± SD. All calculations were performed using SAS statistical software package. Results were considered statistically significant at $p \leq 0.05$.

Student's t test was used for statistical comparison of data from the patients and the control group.

Results

The clinical characteristics of the patients without metabolic syndrome are shown on Table 1. It should be noted that the level of total cholesterol LDL cholesterol and triglicerid were higher in patients with AMI than that of the patients without myocardial infarction. Patients with metabolic syndrome could be characterized by elevated triglicerid, low HDL cholesterol and high LDL cholesterol (Table 2.).

eNos Polymorphism

118 individuals with acute myocardial infarction (defined as a history of chest pain, significant changes in serum creatin kinase) provided blood samples for genotyping of eNOS genes. Blood was also collected for DNA analysis from healthy control group for general practices.

The **eNOS G298T polymorphism** has been detected by DNA melting courve analysis. Representative samples of the polymorph gene variants of eNOS 298 gene is shown on Fig. 1 including 298 GG wt GT heterozygote and TT mutant forms, as well. The specificity of the DNA melting courve analysis has been confirmed by DNA sequencing analysis. The DNA sequence of eNOS 298 GG wt genotype can be seen on Fig. 2a. The DNA sequence of the heterozygote GT genotype of eNOS 298 is shown on Fig. 2b.

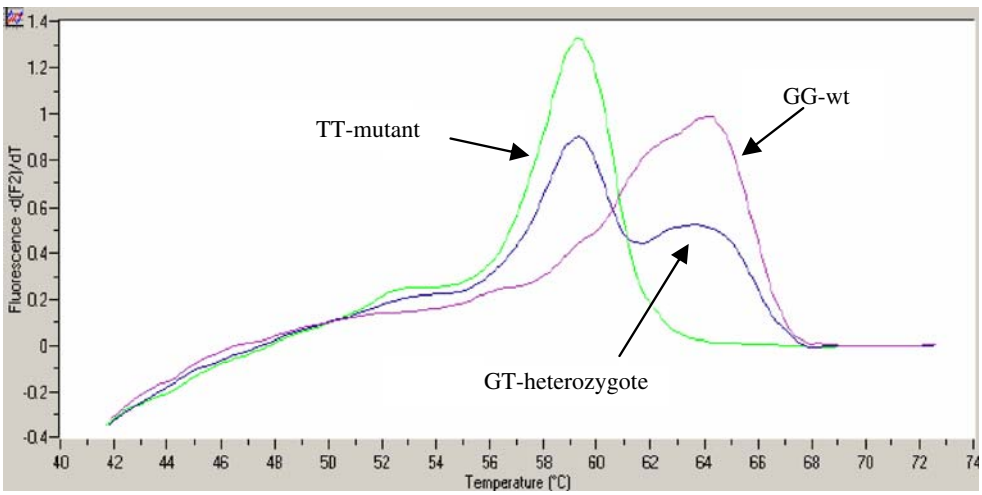

Fig. 1 DNA melting courve analysis of eNOS G 298 T polymorfism

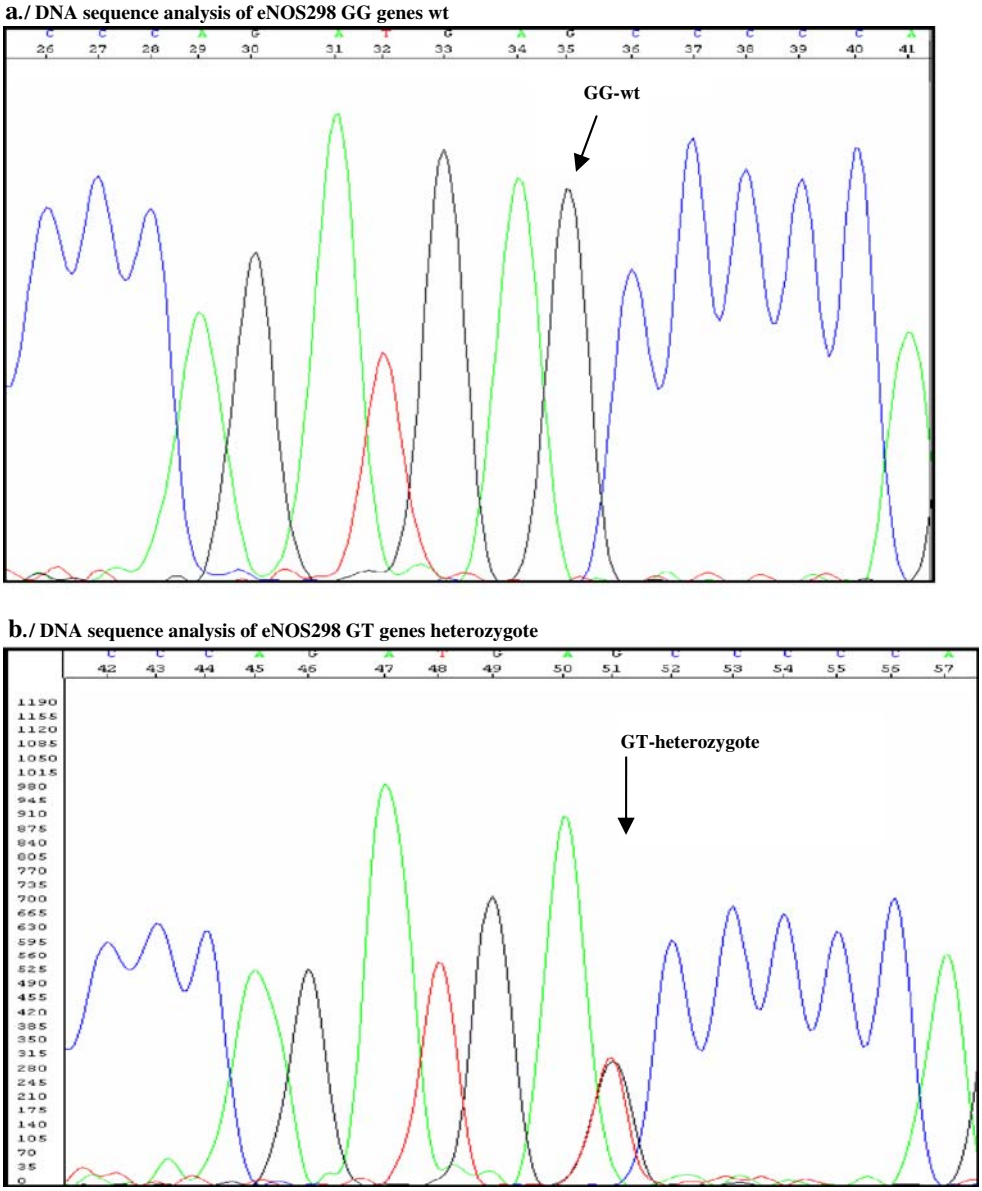

Fig. 2 (**a**) DNA sequence analysis of eNOS298 GG genes wt, (**b**) DNA sequence analysis of eNOS298 GT genes heterozygote

Based on the DNA melting courve analysis the distritubion of various eNOS genotype in patients with or without diabetes is summarized in Table 3.

The allele frequency of wild type eNOS was found to be 52,2% in the healthy control population. At the same time of frequency of wild type eNOS decreased in patient with metabolic syndrome to 41,0%.

Patients without diabetes could be characterized by relatively low level of wild type eNOS 298 that is 33,5% (Table 3.).

Table 3 Correlation between eNOS 298 Glu/ASP genepolymorphism and diabetes, myocardial infractus and stroke

Study group	eNOS 298 ASP/ASP TT %	eNOS 298 Glu/ASP GT %	eNOS 298 Glu/Glu G/G %
1. Control n=384	5,8	42	52,2
2. Diabetes with MI N=101	14,3	44,7	41,0
3. Diabetes with stroke N=108	13,7	43,2	43,1
4. CAD with MI N=118	17,6	48,9	33,5
5. CAD with Stroke	15,2	47,6	37,2

The frequency of eNOS 298 TT mutant genotype is the highest in the atherosclerotic patient with myocardial infarctus (M) $P<0,005$

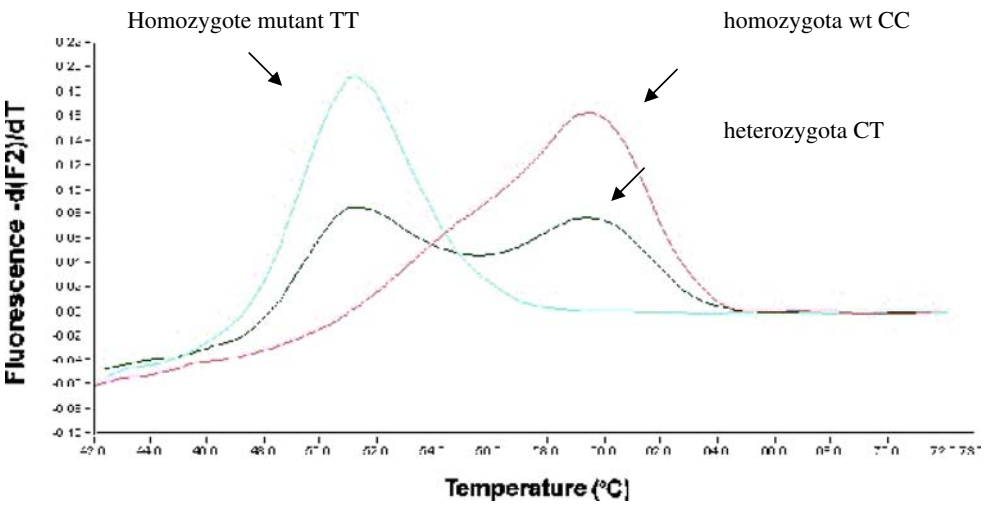

Fig. 3 Melting cure analysis of MTHFR polymorphism C677C → T

The mutant genotype (eNOS 298 TT) was found to be highest in atherosclerotic patient that is 17,6%.

The frequency of eNOS 298 TT genotype in the control subjects was only 5,8%.

MTHFR Polymorphism

The **MTHFR C677T polymorphism** has been identified in atherosclerotic patient with myocardial infarction os stroke. We also analyzed the allele frequency of MTHFR TT, CT and CC genotype in patients with metabolic syndrome associated with myocardial infarction and stroke.The analysis of MTHFR C67T polymorphism has been carried out by DNA melting curve analysis.

Representative example of MTHFR C67T polymorphism is shown on Fig. 3.

The correlation between allele frequency of MTHFR C677T C/T genes stroke and myocardial infarctus is summarized Table 4. It was found that the allele frequency of mutant TT genotype is 11% in the control population. The frequency of these 677 TT mutant genotype of MTHFR gene increased to 21% in patient with cardiovascular disease. The allele frequency of MTHFR genotype also increased in diabetic patient with myocardial infarction up to 17%.

The prevalence of wildtype (C/C) in MI patients (24.0%) was significantly lower ($P<0.01$) than in the control group (57.0%). The prevalence of heterozygous and homozygous variants (C/T genotype, T/T genotype) in MI patiens was also higher ($P<0.05$) than in controls.

Our result demonstrated a positive correlation between the frequency of 677 T allele of MTHFR genes and myocardial infarction and stroke.

Discussion

Methylene Tetrahydrofolate Reductase (MTHFR)

Previous meta-analysis provided conclusive evidence that supports an association between **MTHFR** 677 C/T polymorphism and atherosclerosis [15] via increasing in plasma homocystein (He) level.

Homocystein may promote atherosclerosis and thrombosis by enhancing vascular cell proliferation and promoting prothrombotic activity in the vessel wall [16]. The mutant genotype, TT alleles at position 677 codon of MTHFR gene were reported to associated more frequent stenosis [17–19]. At the same time other studies revealed no association between MTHFR 677TT allele occurance and carotid atherosclerosis [20, 21].

Therefore we examined the frequency of C677T/ MTHFR in a general population with various phenotypes.

Table 4 Correlation between allele frequency of MTHFR C677 C/T genes stroke and myocardial infractus

Study group	MTHFR TT Mutant %	MTHFR CT Heterozygote %	MTHFR CC Wild type %
Control n=384	11	32	57
Diabetes with MI n=101	17	53	30
Diabetes with stroke n=108	15	52	33
CAD with MI n=118	21	55	24
CAD with Stroke	20	54	26

Frequency of MTHFT C677 C/T mutant genotype is more frequent in atherosclerotic patient with MI. $p<0,005$

Our study was also designed to assess the frequency of the *MTHFR* G677T allele in subjects with carotid atherosclerosis.

Our present findings suggest that the A677T allele of *MTHFR* is a genetic risk factor for carotid artery stenosis. Whether this is linked to H(e) levels is not clear, since these levels were unavailable for study and the lack of a gene dose effect would seem to argue against the effect of the A677T allele in MI directly through H(e) levels. The controls in the present study were selected for the absence of atherosclerosis, which is significant in light of the 20% to 30% CAS expected in the general population of this age. These results are compatible with the *MTHFR* A677T allele being a risk factor for atherosclerosis, because its frequency in the controls is lower than in unselected controls and highest in patients with significant CAS.

The cholesterol concentration was not found to be associated with concurrently determined carotid artery atherosclerosis. From the present relatively small study, it is not clear whether A677T is an independent risk factor for MI. There was, however, no significant correlation between the *MTHFR* genotypes and the other examined vascular risk factors. It is especially relevant that the A677T allele was a risk factor for MI. Although the sample size of the present study was sufficient to confirm the prestudy hypothesis that *MTHFR* genotype may influence CAS, a much larger study is needed to assess the interplay between genetic and environmental factors.

In conclusion, our results indicate that the *MTHFR* G677T allele is significantly associated with MI. *MTHFR* 677 G/T genotyping may be of clinical importance as a prognostic and therapeutic marker, although further studies are needed to substantiate this hypothesis.

eNOS Polymorphism

We found an association between genetic variation in the **eNOS** gene and AMI. The association was present in both diabetic and nondiabetic individuals, suggesting and important role of the NO pathway on coronary independent of diabetes status.

NO-dependent endothelial dysfunction is now accepted as a key initial step in atherothrombogenesis. NO is produced in the vasculature by the constitutive endothelial isoform of the nitrix oxide synthases (eNOS).

It has been shown that subject homozygous for the Asp 298 allele generate low NO and may be more susceptible to endothelial dysfunction, which might account for the increased risk for atherosclerosis [22, 23].

A G/T base-pair change at position 894 in exon 7 predicts a Glu298Asp substitution, which is the only polymorphism that alters the primary structure of the protein. This amino acid change influences enzyme stability. Numerous studies have attempted to find a role for this polymorphism in atherothrombotic disease, and similar to findings with the promoter-786 polymorphism, most positive studies have been shown an ≈ 2-fold increase in risk of MI and hypertension was found in carriers of the 298Asp isoform [24].

The major finding of this study was a strong association between a $Glu^{298\rightarrow}$ Asp polymorphism of the eNOS and the risk of CAD. This increased risk was confined to individuals homozygous for the Asp^{298} variant, amounted to a >4-fold risk of CAD compared with individuals homozygous for Glu^{298}. A significant excess of the Asp^{298} homozygotes was also seen among individuals with recent acute MI when compared with healthy controls.

Because NO is considered to be atheroprotective, the excess risk of CAD or MI among Asp^{298} homozygotes may reflect a reduction in the amount or activity of endothelial nitric oxide synthase among such subjects [25].

In summary, we have identified polymorphisms in the *NOS 3* gene and one of these polymorphisms, $Glu^{298\rightarrow}$Asp, was found to be a major risk factor for CAD. This finding is potentially important but requires further confirmation in other populations.

Numerous gene polymorphism associated with the development of coronary and carotid atherosclerotic disease have been identified.

Although individual single nucleotid polymorphism identification provide a framework for genetic susceptibility, the characterization of single gene polymorphism is insufficient to describe the overall effects of genetics on the phenotypic state of atherosclerosis.

There is a need for future studies to take into account gene-gene interaction when using gene profile for prediction of atherosclerosis plaque formation.

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