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QUARTERLY

Carrier-state of D allele in *ACE* gene insertion/deletion polymorphism is associated with coronary artery disease, in contrast to the C677→T transition in the *MTHFR* gene★✪

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Angiotensin I-converting enzyme (ACE), which plays an important role in blood pressure regulation, and methylenetetrahydrofolate reductase (MTHFR) involved in homocysteine metabolism belong to a large group of polypeptides which may be potential risk factors for atherosclerosis and coronary artery disease (CAD). To assess whether polymorphisms of the genes encoding these peptides are associated with CAD in Silesian we conducted a study among 68 individuals suffering from CAD (including 52 cases after myocardial infarction), 51 subjects with positive family history of CAD and 111 controls. We analysed the distribution of genotypes and allele frequencies of the insertion/deletion (I/D) polymorphism in the *ACE* gene using PCR amplification, and the C677→T polymorphism in the *MTHFR* gene using PCR-RFLP analysis.

We found that D allele frequency was significantly higher in CAD patients (61%) than in controls (43%) (P = 0.001, OR = 2.06). The D allele carriers (DD + ID genotypes) were more frequent in the CAD patients (85%) compared to control group (65%) (P = 0.003, OR = 3.14), whereas the familial CAD risk group shows the highest

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Abbreviations: ACE, angiotensin converting enzyme; BMI, body mass index; CAD, coronary artery disease; I/D, insertion/deletion; MI, myocardial infarction; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

frequency of the ID genotype (57% *vs* 43% in controls). In contrast, the *MTHFR* polymorphism does not seem to be associated with the disease.

Our data indicate that in Silesian CAD patients the disease is strongly associated with carrier-state of the *ACE* D allele, but not with the C677→T transition in the *MTHFR* gene.

Coronary artery disease (CAD) is a multifactorial disease caused by various genetic and environmental factors involved in the pathogenesis of atherosclerosis and its thrombotic complications (Marian, 1997). Inflammation plays a key role in susceptibility to coronary atherosclerosis (Libby *et al.,* 2002). The pathogenesis of atherosclerosis is complex and according to response-to-injury hypothesis this process is caused by endothelial damage (Ross, 1999), therefore the contribution of several groups of genes is possible. Some genes encoding "disease markers" may have variants which show differences in expression and may be potential risk factors for atherosclerosis. Particularly, products of genes responsible for the functioning of endothelial cells, macrophages, vascular smooth muscle cells as well as polypeptides involved in regulation of lipid and homocysteine metabolism, blood coagulation and fibrinolysis and hypertension are likely to play a significant role in predisposition to atherosclerosis and its cardiovascular complications (Ballantyne & Abe, 1997; Cambien, 1999). However, interactions between genetic and environmental factors influence progression of pathological processes, clinical characteristics of disease and susceptibility to therapeutical treatment. The conventional risk factors for CAD are hypercholesterolemia or hyperlipidemia, hypertension, overweight or obesity, diabetes mellitus, cigarette smoking and positive family history for coronary artery disease. Genetic factors play an important role in the susceptibility to these disorders. The knowledge of genetic factors of coronary artery diseases may help in explaining the molecular bases of the disorder and in designing prevention and treatment methods, as more than 50% of patients suffering from myocardial infarction have no identifiable conventional risk factor. We took up studies of genetic factors implicated in the susceptibility to atherosclerosis and its cardiovascular complications.

Angiotensin converting enzyme (ACE) is a major component of the renin-angiotensin system and plays an important role in blood pressure regulation by hydrolysing angiotensin I into angiotensin II, a strong vasopressor. This enzyme is also able to degrade bradykinin which is a potent vasodilator (Ruiz-Ortega, 2001). ACE plasma activity is related to the insertion/deletion (I/D) polymorphism of the *ACE* gene (Rigat, 1990). Several studies have shown that the DD genotype is associated with a higher risk for myocardial infarction (MI) and CAD (Arbustini, 1995; Cambien, 1992). However, other studies have failed to show any association between the DD genotype and CAD (Trzeciak, 1996).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme which plays an important role in homocysteine metabolism by catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is the major circulatory form of folate and carbon donor for remethylation of homocysteine to methionine. The cytosine to thymine substitution at nucleotide 677 in the *MTHFR* gene converts alanine to valine at position 226 in the polypeptide which is encoded by this gene (Frosst *et al.,* 1995). This mutation (677C→T) results in a decrease of the enzyme activity that leads to mild hyperhomocysteinemia. Elevated plasma level of homocysteine has been recognized as an independent risk factor for cardiovascular disease (Clarke *et al.,* 1991).

The aim of the present study was to assess the frequencies of the *ACE* and *MTHFR* polymorphisms and to investigate their associations with coronary artery disease and myocardial infarction.

MATERIALS AND METHODS

Subjects. Among 230 subjects included in this study, there were 68 patients (age 32–57) with angiographically documented CAD, including 52 cases who had suffered from myocardial infarction. Some of them underwent therapeutic percutaneous coronary intervention, such as coronary artery by-pass grafting (10 patients) or intracoronary stent (19 subjects). The angiographic inclusion criterion was more than 50% diameter stenosis of at least one major coronary vessel. Patients were included irrespective of concomitant risk factors for atherosclerosis such as hypertension, diabetes mellitus, body mass index (BMI) and cigarette smoking. There were 41 patients with hypertension, 6 with diabetes mellitus, 17 with overweight or obesity and 18 cigarette smokers. This cases group was compared with 111 healthy individuals without a positive family history for MI (age 18–55) in interview, recruited from blood donors. Coronary angiography was not performed on these individuals and therefore the presence of atherosclerotic coronaries cannot be excluded. We also analyzed 51 persons with a positive family history for CAD (age 7–52) in interview. They were subjects closely related (children, sibs) with persons who had suffered from MI. Coronary angiography was not performed on these individuals and therefore the presence of atherosclerotic coronaries cannot be excluded. All patients and volunteers were white Polish Caucasians. The BMI was calculated as mass in kilograms divided by the square of height in meters. The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice and written consents from the patients were obtained.

Biochemical analyses. All examined individuals were instructed to fast for 12–24 h before blood collection. Antecubital venous blood was collected and samples were centrifuged within 2 h of being drawn. Only fresh blood serum was used in the study. Total serum cholesterol, HDL-cholesterol and triglycerides were measured by enzymatic methods (commercial Analco Kit). The coefficients of variation between the measurements for total cholesterol, HDL-cholesterol and triglycerides were 1.5%, 3.9% and 2.0%, respectively. LDL-cholesterol levels were calculated according to the Friedewald formula (Friedewald *et al.,* 1972) in subjects with triglyceride levels below 4.4 mmol/l.

Analyses of polymorphisms. Genomic DNA was extracted from peripheral lymphocytes using MasterPure genomic DNA purification kit (Epicentre Technologies). The *ACE* I/D polymorphism was genotyped using polymerase chain reaction (PCR). Specific primers were described previously (Cambien *et al.,* 1992), but amplification parameters were modified (initial 5 min denaturation at 94°C, 30 cycles with 1 min at 92°C, 1 min at 52°C, 2.5 min at 72°C and 6 min final extension at 72°C). The PCR products were 490 bp for allele I and 190 bp for allele D (Fig. 1a). Products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

For the identification of the 677C→T mutation in the *MTHFR* gene we used PCR-RFLP (restriction fragment length polymorphism) analysis. Genomic DNA was amplified with specific primers described by Frosst *et al.* (1995). The mixture was denatured initially at 96°C for 5 min, followed by 35 cycles with 50 s at 93°C, 50 s at 60°C, 30 s at 72°C and 7 min final extension at 72°C. Then, the PCR product (198 bp) was digested with 2.5 U of *Hin*f I (Promega) restriction enzyme. The mutation 677C→T creates a *Hin*f I recognition sequence and the product is digested into 175 bp and 23 bp fragments (Fig. 1b). The products of restriction were separated on a 3% agarose gel and visualized by ethidium bromide staining.

Statistical analyses. The data were analyzed using the *STATISTICA* 6.0 software. Allele frequencies were deduced from the genotype distribution. Hardy-Weinberg equilibrium was tested in all groups by a χ^2 test. Comparisons of genotype and allele frequencies between cases and control subjects were

performed by a χ^2 test with Fisher's correction. Statistical significance was accepted at $P < 0.05$. Odds ratios (OR) as well as their 95% Cl were computed to assess the strength of the association between the presence of the polymorphic alleles and genotypes, and CAD.

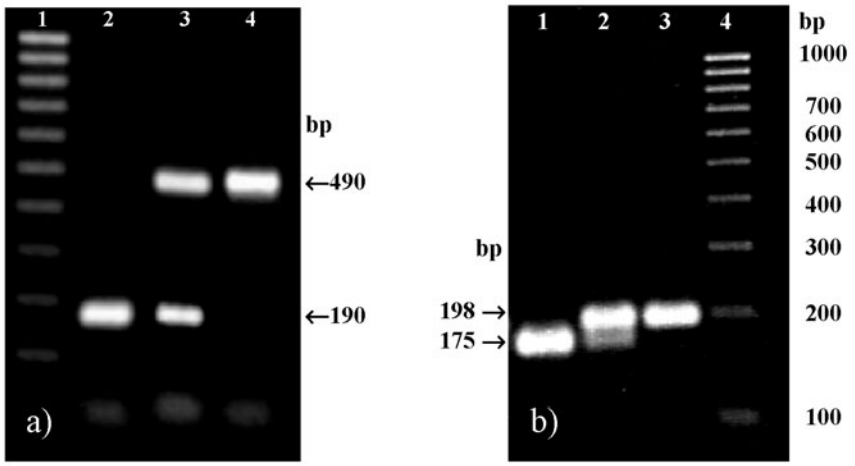

Figure 1. Detection of *ACE* I/D polymorphism.

a) 1, 100–1000 bp DNA ladder; 2, DD homozygous; 3, ID heterozygous; 4, II homozygous, and *MTHFR Hinf* I polymorphism; b) 1, homozygous TT; 2, heterozygous CT; 3, homozygous CC; 4, 100–1000 bp DNA ladder.

RESULTS AND DISCUSSION

Clinical and biochemical parameters like mean age, BMI, total serum cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides of CAD patients, control and subjects with a positive familial history for CAD are shown in Table 1. Among the CAD patients the percentage of females is slightly higher than in controls and the mean value of the age is also a little higher in this group. Mean BMI is similar in the patients and controls, only in the familial risk group it is lower due to young age. The CAD patients show increased level of total cholesterol and LDL-cholesterol, which was predictable since these two parameters belong to traditional risk factors of CAD. However, surprisingly, the mean value of HDL-cholesterol, which is a known protective factor, is also the highest among the cases. This is probably due to the pharmaceutical treatment of most of the patients (statins, fibrates) or the relatively low mean value of HDL-cholesterol in controls, which may be associated with the general tendency for increased total and LDL-cholesterol serum levels and decreased HDL-cholesterol level observed in the Polish population.

The allele and genotype frequencies of the analyzed gene polymorphisms for all groups are reported in Table 2. The genotype frequencies among the cases, control subjects and the familial CAD risk group for both analyzed genes were compatible with Hardy-Weinberg equilibrium.

The estimated frequencies of the alleles and genotypes were compared between the CAD patients, familial CAD risk group and controls. Comparisons of the genotype and allele frequencies between the groups, performed by a χ^2 test, and odds ratios for CAD associated with the presence of the alleles and genotypes of *ACE* polymorphism are shown in Table 3.

ACE

The greatest difference in the allele frequencies and genotype distribution between the subjects and controls we found for the

Table 1. Clinical and biochemical characteristics of the study groups

	CAD patients	Familial CAD risk subjects	Control subjects
Number of subjects	68	51	111
women	17 (25%)	24 (47%)	15 (13%)
men	51 (75%)	27 (53%)	97 (87%)
Mean age (years ± S.D.)	46 ± 6.5	26 ± 12.9	35 ± 9.9
Mean BMI (kg/m^2 ± S.D.)	26.5 ± 4.09	22.8 ± 5.05	25.2 ± 3.94
Total serum cholesterol (mmol/l ± S.D.)	5.6 ± 1.27*	4.74 ± 0.92	4.9 ± 2.2
Serum HDL (mmol/l ± S.D.)	1.18 ± 0.37*	1.02 ± 0.3*	0.89 ± 0.30
Serum LDL (mmol/l ± S.D.)	3.64 ± 1.0*	3.15 ± 0.86	3.32 ± 2.04
Serum TG (mmol/l ± S.D.)	1.89 ± 1.0*	1.18 ± 0.68*	1.32 ± 0.51

±S.D., standard deviation; *Statistically significant data ($P < 0.05$)

I/D polymorphism of the *ACE* gene. The D allele frequency was 18% higher in the cases than in controls (61% *vs* 43%), and the difference was statistically significant ($P = 0.001$, OR = 2.06). The DD genotype was more frequent in CAD patients compared to control subjects (37% *vs* 22%), and again this was significant ($P = 0.027$). However the relative risk for DD genotype was high (OR = 2.11), this data was not statistically significant (CI 0.56–7.91). The aggregate frequency of D allele carriers (subjects with ID and DD genotypes) was 20% higher in the patients than in controls (85% *vs* 65%, $P = 0.003$, OR = 3.14; CI 1.30–7.29). In contrast the frequency of the II genotype was more than twice higher in controls than in the patients (35% *vs* 15%). The ID genotype was more frequent in the familial CAD risk group than in the control group (57% *vs* 43%) and than in the cases (57% *vs* 48%), but these differences were not statistically significant.

Table 2. The frequency of genotypes and alleles of the analyzed polymorphisms in CAD patients, familial CAD risk group and controls

GENE		GENOTYPE				ALLELE	
ACE		II	ID	DD	ID+DD	I	D
CAD patients	n	10	33	25	58	53	83
	Frequency	0.15	0.48	0.37	0.85	0.39	0.61
Familial CAD risk group	n	10	29	12	41	49	53
	Frequency	0.20	0.57	0.23	0.80	0.48	0.52
Control group	n	39	48	24	72	126	96
	Frequency	0.35	0.43	0.22	0.65	0.57	0.43
MTHFR		CC	CT	TT	CT+TT	C	T
CAD patients	n	29	31	6	37	89	43
	Frequency	0.44	0.47	0.09	0.56	0.67	0.33
Familial CAD risk group	n	19	29	3	32	67	35
	Frequency	0.37	0.57	0.06	0.63	0.66	0.34
Control group	n	47	56	8	64	150	72
	Frequency	0.42	0.50	0.08	0.63	0.68	0.32

Results of previous studies on the *ACE* I/D polymorphism association with cardiovascular diseases were inconsistent. In the first publication associating the I/D *ACE* polymorphism with myocardial infarction, Cambien and coworkers (Cambien *et al.,* 1992) reported that the D allele of the *ACE* gene is a potent risk factor for myocardial infarction. They analyzed genotype and allele distribution of the I/D polymorphism in a few European populations. In the population of Lille (France) the DD genotype frequency was 17% higher in patients after MI than in control subjects. Cambien *et al.* (1992) found a statistically significant relationship between the DD genotype and MI in this population ($P = 0.007$). After that publication many researchers tried to confirm those data. In the Italian population Arbustini and coworkers (Arbustini *et al.*, 1995) found a relationship between the D allele presence and coronary atherosclerosis and myocardial infarction. Those authors think that the *ACE* deletion genotype is strongly and independently associated with coronary atherosclerosis (OR = 5.78) and, to a lesser extent, with MI (OR = 2.56). Ruiz *et al.* (1992) analyzed frequencies of alleles and genotypes of the *ACE* I/D polymorphism in 132 non-insulin-dependent diabetes mellitus (NIDDM) who had myocardial infarction and 184 NIDDM patients without history of CAD. They found that the D allele was a strong and independent risk factor for CAD in the NIDDM patients. The increased relative risk for the DD genotype was OR = 2.35. Beohar *et al.* (1997) analyzed white Americans. The *ACE* DD genotype was present in 47% of patients with coronary artery disease compared to 30% in general population ($P = 0.0002$, OR = 2.7). They concluded that the *ACE* deletion polymorphism is a genetic risk factor for CAD. Wang and coworkers (1996) analyzed the distribution of the I/D *ACE* polymorphism genotypes in white Australians. They found statistically important differences in the DD genotype frequency among patients with CAD compared to healthy school children ($\chi^2 = 23.69$, $P < 0.0001$).

Table 3. Comparison of genotype and allele frequencies between cases and control subjects using χ^2 test

GENE	ALLELE	GENOTYPE	
ACE	D	DD/ID+II	ID+DD/II
χ^2 (p)	10.67 (P=0.001)*	4.86 (P=0.027) *	8.85 (P=0.0029)*
Fisher's p			
one-way	P=0.0008*	P=0.022*	P=0.002*
two-ways	P=0.0015*	P=0.038*	P=0.003*
OR	2.06*	2.11	3.14*
(95% CI)	(1.01–4.16)	(0.56–7.91)	(1.30–7.29)

*Statistically significant data

On the other hand, no association between the DD genotype and MI was found by Bohn *et al.* (1993) in Norwegians. The DD genotype frequency was higher in controls than in patients (38.3% *vs* 20.9%). In conclusion of a meta-analysis based on a literature search of MEDLINE up until April 1998 (Agerholm-Larsen *et al.*, 2000), the *ACE* gene I/D polymorphism affects plasma ACE activity but is not associated with increased risk of coronary artery disease and myocardial infarction.

Our publication is not the first study in Poland which investigate the I/D *ACE* polymorphism association with cardiovascular diseases. Trzeciak and coworkers (1996) found no association between the D allele and myocardial infarction in the population of Poznań. Gorący (2000) reported that there is no associ-

ation between the *ACE* I/D polymorphism and myocardial infarction in the Szczecin population. Thus the *ACE* I/D polymorphism seems to be a potential risk factor only in Silesian CAD patients. These differences may result from specificity of population (regional environmental influences) or selection of study groups.

MTHFR

We did not find evident differences between the patients and controls in the allele frequencies and genotype distribution of the C677→T *MTHFR* gene polymorphism. The T and C allele frequencies in the cases and controls were the same. The genotype distribution in the analyzed groups was similar. Although we observed that the TT genotype frequency was higher in the CAD subjects compared to controls (9% *vs* 8%) and compared to the familial CAD risk group (9% *vs* 6%), these differences were not statistically significant.

Some authors suggest that the C677→T mutation in the *MTHFR* gene is a potential risk factor for cardiovascular events. Frosst and coworkers (1995) reported that this mutation in the heterozygous or homozygous state correlates with reduced enzyme activity and increased thermolability in lymphocyte extracts. In contrast, Brugada and Marian (1997) determined the frequency of the C677→T genotypes and alleles in Caucasian patients with angiographically documented CAD and in normal individuals. The *MTHFR* TT genotype was not found to be a major genetic risk factor for predisposition to CAD in this population. Gulec and coworkers (2001) found a relationship between the C677→T transition in the *MTHFR* gene and myocardial infarction in Turkish men. The relative risk of MI for TT genotype carriers was 5.94 (95% CI, $P = 0.0016$). Also in the Japanese population the TT genotype increases plasma levels of homocysteine (Nakai *et al.,* 2000) and is an independent risk factor for coronary heart disease (Ou *et al.,* 1998) and myocardial infarction (Nakai *et al.,* 2000). We found only one paper about the *MTHFR* polymorphism and MI in Polish subjects by Gorący (2000). Our study is in agreement with those data reporting, that there is no association between the *MTHFR* C677→T polymorphism and myocardial infarction in the Szczecin population.

Our data indicate that in Silesian CAD patients the disease is strongly associated with carrier-state of the *ACE* D allele, but not with the C677→T transition in the *MTHFR* gene.

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