THE TT GENOTYPE OF THE MTHFR 677C>T POLYMORPHISM INCREASES SUSCEPTIBILITY TO PREMATURE CORONARY ARTERY DISEASE IN INTERACTION WITH SOME OF THE TRADITIONAL RISK FACTORS

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Summary: Background: The presence of several risk factors (genetic and non-genetic) has greater impact on the risk of premature coronary artery disease (CAD) than single risk factor. Objective: The aim of the study was to establish possible relations between genotypes and alleles of 677C>T polymorphism of *MTHFR* gene and some traditional risk factors e.g. elevated levels of lipid parameters and smoking in development of premature CAD. Methods: The groups comprised 152 patients with angiographically documented premature CAD (aged 42.9 ± 5.5) and 121 age-matched blood donors (aged 42.3 ± 6.5) were studied. The *MTHFR* 677C>T polymorphism was genotyped with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. Results: Patients with TT genotype who simultaneously smoked had increased risk of premature CAD compared to non-smoking cases with CC genotype (OR = 24.62). We also found that individuals with TT genotype and elevated LDL-cholesterol (LDL-chol.) level had significantly higher risk of CAD (OR = 9.92) than individuals with normal LDL-chol. level and CC genotype. Conclusions: The present study shows that simultaneous presence of *MTHFR* TT genotype and smoking or elevated levels of LDL-chol. influences the risk of premature CAD. This findings give interesting contribution to gene-environment interaction problem that may have clinical implications in the future.

Key words: *Coronary artery disease; Polymorphism; MTHFR; Lipids; Traditional risk factors*

Introduction

Coronary artery disease has a complex etiology generated by combined effects of both, genetic and environmental factors (1). The polymorphic genes, encoding products involved in atherosclerotic process, predispose individuals to a greater or lower extent to CAD. However, traditional risk factors, such as cigarette smoking, hypercholesterolemia, hypertension and overweight, interacting with the genetic risk factors (in cumulative or synergistic ways), may increase or not the risk of the disease. It is known that interactions between genetic and environmental factors are very important in subjects with a high-risk genetic profile (2). Genetic factors have greater contribution to the development of CAD at younger age (3).

Methylenetetrahydrofolate reductase (MTHFR) catalyzes a reduction of 5,10-methylenetetrahydrofolate to 5-methyletetrahydrofolate that is the carbon donor for the remethylation of homocysteine (Hcys) to methionine (4). The 677C>T polymorphism in the *MTHFR* gene influences enzyme thermolability that leads to its decreased activity and to elevated level of plasma Hcys. Since hyperhomocysteinemia causes a chronic inflammatory state it is considered to be a risk factor for CAD, stroke and thrombosis (5–7). Thus, also the 677C>T polymorphism may be considered as a risk factor for CAD (4, 8).

The objective of the present study was to assess possible relations between genotypes and alleles of 677C>T polymorphism of *MTHFR* gene and some traditional risk factors e.g. elevated levels of lipid parameters, smoking and overweight in the development of premature CAD.

Materials and methods

Patients and controls

The study population consisted of 273 subjects, white Polish Caucasians, inhabitants of Upper Silesia region (Katowice, Poland).

Group 1 (CAD) comprised 152 unrelated patients with premature CAD confirmed by angiography with more than 50% diameter stenosis of at least one of the major coronary vessels (64 women and 88 men, mean age 42.9 ± 5.5).

Group 2 (Control) comprised 121 age-matched healthy blood donors with no signs of CAD in interview (19 women and 102 men, mean age 42.3 ± 6.5).

The patients were recruited from the 1st Clinic of Cardiology in the Silesian Center of Cardiology in Katowice during the period 2000–2004. The coronary angiography was performed by means of Judkin's method. Myocardial infarction (MI) was diagnosed according to recommendations of the Joint European Society of Cardiology/American College of Cardiology Committee (9). The exclusion criteria from the study was: clinical diagnosis of cardiomyopathy, coagulopathy, collagenoses and acute poisoning (e.g. CO, amphetamine). Individuals with chronic inflammatory or autoimmune disease and any kind of dementia were also excluded. Cardiomyopathies were detected by electrocardiogram, echocardiography and coronarography. Stroke was diagnosed by computed tomography and magnetic resonance imaging. Other diseases, including peripheral artery occlusive disease, were diagnosed on the basis of medical interview, examination and laboratory methods.

Blood donors were recruited from Regional Center of Blood Donor and Blood Treatment in Katowice. The exclusion criterion was CAD or stroke revealed in the course of family history. CAD in this case was defined through its occurrence in at least one of the parents.

The entire patients' group was characterized in respect of concomitant risk factors for atherosclerosis such as hypertension, hyperlipidemia, cigarette smoking, overweight/obesity and diabetes mellitus on the basis of medical interview according to previously described standards (10).

The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice and all subjects gave written informed consents.

Biochemical analyses

Antecubital venous blood was collected from each participant after an overnight fast, immediately put on ice and transferred to the laboratory for serum separation within 2 hours after being drawn. Only fresh blood serum was used in the study. The levels of lipid parameters like total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-chol.) and triacylglycerols (TG) were measured using enzymatic methods (commercial Analco Kit, Warsaw, Poland). The concentration of LDL-chol. was calculated according to the Friedewald formula in subjects with levels of triacylglycerols below 4.4 mmol/l (11).

Analyses of polymorphisms

Genomic DNA was extracted from peripheral lymphocytes using commercial MasterPure genomic DNA purification kit (Epicentre Technologies; Madison, WI, USA). The 677C>T polymorphism of *MTHFR* was analyzed using PCR-RFLP method. Genotyping of the *MTHFR* polymorphism was carried out as described previously by Frosst et al. (4), with some modification of the amplification parameters, which were as follows: 5 min of initial denaturation at 96 °C, 35 cycles with 35 s of denaturation at 93 °C, 5 s of annealing at 60 °C, 30 s of extension at 72 °C and 7 min of final extension at 72 °C. The PCR product, length 198 base pairs (bp), was digested by *Hinf*I restriction enzyme (Promega; Madison, WI, USA) generating fragment 198 bp for genotype CC; fragments: 198, 175 and 23 bp for genotype CT and 175, 23 bp for genotype TT. To avoid genotyping errors all restriction analyses were done in the same conditions and with the *Hinf*I restriction enzyme coming from the same source. The CC homozygotes were considerend as wild type genotype in the whole manuscript.

Statistical analyses

Data were analyzed using the *Statistica 7.1* (STATSOFT; Statistica, Tulsa, OK, USA) and the *EpiInfo 6* (Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA) softwares. Normality of distribution of quantitative data was computed by Shapiro-Wilk *W* test. The comparison of mean values of quantitative data between all study groups was made using two following tests: the Student's *t*-test was used when the distribution of some data was normal and Mann-Whitney *U* test, when the distribution of quantitative data differed from normal distribution. Allele frequencies were assessed on the basis of the genotype distribution. Hardy-Weinberg equilibrium (HWE) in all groups was tested by a χ^2 test. The comparisons of genotype and allele frequencies between cases and controls was also assessed with the χ^2 test. In case of small number of subjects (< 5) in the analysis, the Fisher's correction was used. Statistical significance was accepted at $p < 0.05$. To assess an association between alleles or genotypes and the disease odds ratios (OR) as well as their 95% confidence interval (CI) were computed. The relationships between polymorphic variants and the disease was calculated using uni- and multivariate logistic regression analysis after adjustment for traditional risk factors, such as: smoking, elevated level of TC, LDL-chol., TG, overweight/obesity. The 6 × 2 table approach was used to assess the additive interactions between the genotypes and traditional risk factors. The reference category involves individuals carrying no polymorphic variant and no traditional risk factor.

Results

General, biochemical and clinical characteristic of the study groups

General characteristics and levels of biochemical parameters like TC, HDL-, LDL-chol. and TG of the study groups are shown in Table 1. Cases had significantly higher levels of TC, LDL-cholesterol and TG compared to control group. The mean body mass index (BMI) value was also greater in patients than in controls. There were greater number of smokers among patients compared to controls (58% vs 24%, $p < 0.001$, OR = 4.48 95% CI 2.56–7.88).

Tab. 1: General characteristics of the study groups

	Patients with premature CAD n = 152	Control group n = 121
Sex (n, %)	♀ 64 (42.1%) ♂ 88 (57.9%)	♀ 19 (15.7%) ♂ 102 (84.3%)
Age (mean ± SD)	42.9 ± 5.5	42.3 ± 6.5
BMI (mean ± SD)	26.7 ± 4.3*	25.4 ± 3.5
Smoking habit (n, %)	89* (58.6%)	29 (24.0%)
Total cholesterol (mean ± SD) mmol/l	5.8 ± 1.4*	5.3 ± 1.4
LDL-cholesterol (mean ± SD) mmol/l	3.9 ± 1.2*	3.5 ± 1.2
HDL-cholesterol (mean ± SD) mmol/l	1.1 ± 0.3	1.1 ± 0.4
Triacylglycerols (mean ± SD) mmol/l	1.9 ± 1.0*	1.5 ± 0.7

CAD-coronary artery disease, LDL-low density lipoproteins, HDL-high density lipoproteins, BMI-body mass index, SD-standard deviation

* statistically significant data at $p < 0.05$

Almost 86% of the cases were recruited to the study in the state of acute coronary syndrome. Among patients there were 81% individuals who suffered from MI and 14.6% of them had more than one MI. Almost 60% of cases had critical stenosis and multivessel disease. More than 50% of patients had arterial hypertension.

Analyses of polymorphism

The genotype and allele frequencies of 677C>T polymorphism of *MTHFR* gene in both groups were compatible to HWE (premature CAD $p = 0.442$, $\chi^2 = 0.59$, control group $p = 0.11$, $\chi^2 = 2.56$). The term "carriers" were used for subjects with at least one T allele (subjects with genotypes CT+TT). The distributions of the *MTHFR* genotypes and alleles in the entire groups are shown in Table 2.

We found that T allele of *MTHFR* gene was more frequent in cases compared to controls (34% vs 28%). It was also observed that homozygotes TT were twice as common in cases (10%) than in controls (5%) in reference to CC homozygotes (OR = 2.71). This difference was on the borderline of statistical significance ($p = 0.083$ in multivariate analysis). The T allele carriers were also more common in the patients' group compared to controls (57% vs 51%).

Tab. 2: Distribution of genotypes and alleles of *MTHFR* polymorphism and the results of comparison between CAD and control in entire groups and male subgroups

		Entire groups				Male subgroups			
Gene	Genotype / allele	Premature CAD n = 152		Control n = 121		Premature CAD n = 88		Control n = 102	
		n	%	n	%	n	%	n	%
MTHFR	CC	65	43	59	49	37	42	49	48
	CT	72	47	56	46	42	48	48	47
	TT	15	10	6	5	9	10	5	5
	CT+TT	87	57	62	51	51	58	53	52
	C	202	66	174	72	116	66	146	72
	T	102	34	68	28	60	34	58	28
T/C p = 0.171, OR = 1.29 95%CI 0.88–1.90* TT/CC p = 0.105, OR = 2.27 95%CI 0.76–7.06* p = 0.083, OR = 2.71 95%CI 0.87–8.43** CT+TT/CC p = 0.323, OR = 1.27 95%CI 0.77–2.12* p = 0.099, OR = 1.51 95%CI 0.92–2.45**						T/C p = 0.235, OR = 1.30 95%CI 0.82–2.06* TT/CC p = 0.139, OR = 2.38 95%CI 0.65–9.04* p = 0.264, OR = 2.05 95%CI 0.58–7.28** CT+TT/CC p = 0.408, OR = 1.27 95%CI 0.69–2.36*p = 0.310, OR = 1.41 95%CI 0.72–2.77**			

CAD-coronary artery disease, OR-odds ratio, CI-confidence interval, * univariate analysis, ** multivariate analysis adjusted with levels of LDL-cholesterol, triacylglycerols and smoking

Relations between MTHFR polymorphism and traditional risk factors

The main goal of our study was to analyze whether there are any interactions between 677C>T polymorphism of the *MTHFR* gene and traditional risk factors which could increase the risk of premature CAD in the Polish patients from Upper Silesia region. There was observed increased risk of premature CAD in patients with CT genotype who simultaneously smoked when compared to non-smoking cases with wild-type genotype (OR = 4.76). The risk was significantly increased in smoking subjects who were TT homozygotes (OR = 24.62). The exact calculations (6 × 2 approaches) of interactions between *MTHFR* genotypes and traditional risk factors are shown in Table 3. Moreover we demonstrated that the susceptibility to premature CAD was increased with the presence of at least one T allele of *MTHFR* polymorphism and elevated level of LDL-chol. Individuals with TT genotype and elevated LDL-chol. level had significantly higher risk of CAD (OR = 9.92) than individuals with normal LDL-chol. level and CC genotype. Similar result was found in case of TT homozygous subjects with contemporaneous presence of overweight (OR = 10.15). These results may suggest that there are some additive relationships between *MTHFR* polymorphism and conventional risk factors for CAD in predicting the risk of the disease at younger age.

Tab. 3: Associations between genotypes of 677C>T polymorphism of *MTHFR* gene and traditional risk factors of CAD in study groups

Genotype	Smoking	CAD	Control	OR (95% CI), p
CC	no	21	47	reference data
CC	yes	44	12	8.21 (3.37–20.39), p < 0.001*
CT	no	38	40	2.13 (1.02–4.45), p = 0.028*
CT	yes	34	16	4.76 (2.02–11.32), p < 0.001*
TT	no	4	5	1.79 (0.36–8.82), p = 0.414 Fisher exact 1-tailed p = 0.321 Fisher exact 2-tailed p = 0.461
TT	yes	11	1	24.62 (2.92–543.53), p = 0.00007* Fisher exact 1-tailed p = 0.0001 Fisher exact 2-tailed p = 0.0001
Genotype	TC > 5.2mmol/l	CAD	Control	OR (95% CI), p
CC	no	24	30	reference data
CC	yes	41	29	1.77 (0.81–3.86), p = 0.118
CT	no	25	28	1.12 (0.49–2.56), p = 0.777
CT	yes	47	28	2.10 (0.97–4.56), p = 0.04*
TT	no	6	3	2.50 (0.48–14.33), p = 0.216 Fisher exact 1-tailed p = 0.199 Fisher exact 2-tailed p = 0.289
TT	yes	9	3	3.75 (0.80–19.88), p = 0.055
Genotype	LDL > 3.4 mmol/l	CAD	Control	OR (95% CI), p
CC	no	24	34	reference data
CC	yes	41	25	2.32 (1.06–5.11), p = 0.021*
CT	no	31	28	1.57 (0.71–3.48), p = 0.226
CT	yes	41	28	2.07 (0.96–4.50), p = 0.043*
TT	no	8	5	2.27 (0.57–9.26), p = 0.187
TT	yes	7	1	9.92 (1.09–228.80), p = 0.0143* Fisher exact 1-tailed p = 0.017 Fisher exact 2-tailed p = 0.021

Genotype	Overweight BMI > 25	CAD	Control	OR (95% CI), p
CC	no	26	33	reference data
CC	yes	39	26	1.90 (0.88-4.15), p=0.076
CT	no	30	26	1.46 (0.66-3.27), p=0.308
CT	yes	42	30	1.78 (0.84-3.79), p=0.104
TT	no	7	5	1.78 (0.44-7.44), p=0.366
TT	yes	8	1	10.15 (1.15-230.41), p=0.012* Fisher exact 1-tailed p=0.014 Fisher exact 2-tailed p=0.027

CAD-coronary artery disease, TC – total cholesterol, LDL-low density lipoprotein, BMI-body mass index, OR-odds ratio, CI-confidence interval
* statistically significant data at $p < 0.05$

Additionally, we have made analysis of associations between *MTHFR* polymorphism and traditional risk factors and CAD in male subgroups due to gender disproportion. Table 4 shows the results of these calculations. We observed increased risk of premature CAD in male patients with CT genotype who simultaneously smoked compared to non-smoking cases with CC genotype (OR = 5.57). The OR could not be defined in case of smoking male patients with TT genotype because of the lack of smoking control subject bearing TT genotype. We also found higher risk of premature CAD in male TT homozygous cases with overweight (OR = 8.33).

Tab. 4: Associations between genotypes of 677C>T polymorphism of *MTHFR* gene and traditional risk factors of CAD in male subgroups

Genotype	Smoking	CAD	Control	OR (95% CI), p
CC	no	10	39	reference data
CC	yes	27	10	10.53 (3.48–33.10), p < 0.001*
CT	no	22	34	2.52 (0.97–6.68), p = 0.036*
CT	yes	20	14	5.57 (1.90–16.74), p = 0.0003*
TT	no	0	5	0.00 (0.00–5.55), p = 0.260 Fisher exact 1-tailed p = 0.343 Fisher exact 2-tailed p = 0.571
TT	yes	9	0	undefined, p < 0.0001* Fisher exact 1-tailed p < 0.0001 Fisher exact 2-tailed p < 0.0001
Genotype	TC > 5.2mmol/l	CAD	Control	OR (95% CI), p
CC	no	15	23	reference data
CC	yes	22	26	1.30 (0.50–3.37), p = 0.554
CT	no	13	22	0.91 (0.32–2.59), p = 0.838
CT	yes	29	26	1.71 (0.68–4.32), p = 0.208
TT	no	5	2	3.83 (0.54–33.31), p = 0.118 Fisher exact 1-tailed p = 0.125 Fisher exact 2-tailed p = 0.214
TT	yes	4	3	2.04 (0.32–13.86), p = 0.384 Fisher exact 1-tailed p = 0.322 Fisher exact 2-tailed p = 0.433

Genotype	LDL > 3.4 mmol/l	CAD	Control	OR (95% CI), p
CC	no	16	28	reference data
CC	yes	21	21	1.75 (0.68–4.55), p = 0.202
CT	no	18	22	1.43 (0.55–3.77), p = 0.421
CT	yes	24	26	1.62 (0.65–4.03), p = 0.255
TT	no	6	4	2.63 (0.54–13.39), p = 0.170 Fisher exact 1-tailed p = 0.155 Fisher exact 2-tailed p = 0.285
TT	yes	3	1	5.25 (0.42–143.19), p = 0.130 Fisher exact 1-tailed p = 0.164 Fisher exact 2-tailed p = 0.286
Genotype	Overweight BMI > 25	CAD	Control	OR (95% CI), p
CC	no	15	25	reference data
CC	yes	22	24	1.53 (0.59–3.97), p = 0.335
CT	no	18	24	1.25 (0.47–3.33), p = 0.621
CT	yes	24	24	1.67 (0.65–4.29), p = 0.240
TT	no	4	4	1.67 (0.29–9.68), p = 0.509 Fisher exact 1-tailed p = 0.390 Fisher exact 2-tailed p = 0.695
TT	yes	5	1	8.33 (0.79–207.83), p = 0.035* Fisher exact 1-tailed p = 0.047 Fisher exact 2-tailed p = 0.071

CAD-coronary artery disease, TC – total cholesterol, LDL-low density lipoprotein, BMI-body mass index, OR-odds ratio, CI-confidence interval
* statistically significant data at $p < 0.05$

Discussion

The 677C>T transition within *MTHFR* gene results in substitution of the alanine into valine in the mature reductase. This change causes increased thermolability and decreased enzymatic activity and in turn the elevation of the HCys level (4). The *MTHFR* polymorphism itself is related to CAD only in non-European populations (12–15) but not in European population (18). Atherosclerosis and CAD are multifactorial disorders. Clinical phenotype of these diseases may result of interactions between various genetic and environmental factors. Single gene analyses confirmed that associations between single genetic factors and CAD were rather weak. In our opinion, the analysis of possible interactions (synergistic, cumulative or antagonistic) between genetic and non-genetic factors may give more plausible results in the assessment of CAD risk in certain population.

Our study indicates that T allele of *MTHFR* 677C>T polymorphism as well as TT homozygotes (in comparison to CC "wild"-type homozygotes) were more common in patients with premature CAD than in controls. We observed insignificant trend to higher frequency of T allele carriers in cases compared to controls. We found that presence of the TT genotype and one of the traditional risk factor for CAD, e.g. elevated level of LDL-chol., smoking or overweight, significantly increased the risk of the disease. Higher risk of premature CAD was observed in carriers of T allele who simultaneously smoke compared to non-smoking cases with wild-type genotype. The risk was especially high in smoking subjects bearing TT genotype (OR = 24.62).

The *MTHFR* 677C>T polymorphism and smoking habit were known to be the main predictors of total HCys levels (15). Previously, significant interaction between smoking and *MTHFR* 677C>T polymorphism in the group of patients with lung cancer was found (16). Surprisingly, the authors demonstrated that CT and TT genotypes of *MTHFR* polymorphism in association with smoking conferred a decreased risk for lung cancer (16). In the large study exploring interactions between smoking and 34 SNPs of 24 genes in atherosclerosis, the 677C>T polymorphism of *MTHFR* gene and smoking significantly interacted in four arteries (17). The authors observed that smokers bearing

TT genotype are more likely to develop atherosclerosis than subjects with CT or CC genotypes.

In present study we also found that individuals with TT genotype and elevated LDL-chol. level had significantly higher risk of CAD (OR = 9.92). In Croatian patients with carotid stenosis the carriers of the T allele had higher levels of LDL-chol. compared to CC homozygotes (18). Yilmaz et al. (19) demonstrated unfavorable effect of *MTHFR* polymorphism on serum lipid profile that leads to an elevated level of LDL-chol. in renal transplant recipients. The TT subjects in this study group had the highest LDL-chol. levels compared to CC or CT genotypes (19). Previously a positive correlation between plasma HCys level and cholesterol was observed in patients with hyperhomocysteinemia and in HepG2 cells (20). Since HCys stimulates the production and secretion of cholesterol and apoB100 in HepG2 cells (20), the *MTHFR* polymorphism related to elevated level of HCys may be indirectly associated to the production of cholesterol. Earlier, an interaction between LDL-chol. and HCys was suggested in the atherosclerotic processes (21).

We demonstrated also a relation between the *MTHFR* TT genotype and contemporaneous presence of overweight (OR = 10.15). Recently, statistically higher levels of serum HCys were found in the overweight or obese individuals compared to subjects with normal BMI (22). There are conflicting results showing associations of *MTHFR* polymorphism and overweight or obesity (23–25). Some studies did not show such relations (23, 24). However, some of them showed higher BMI and waist-to-hip ratio in healthy postmenopausal women being carriers of the 677C>T polymorphism compared to women with the CC genotype (25). On the other hand, in the study of Frelut et al. (26) the highest value of BMI was found in the obese adolescent girls having CC genotype compared to girls with CT or TT.

Our previous data demonstrated a role of *MTHFR* polymorphism in the cardio- and cerebrovascular diseases (27, 28). In the group of patients with CAD we observed that frequency of some double or triple combinations among polymorphisms of the *MTHFR, IL-6* and *ICAM1* genes, particularly for: *MTHFR+ICAM1* and *MTHFR+ICAM1+IL-6* patterns, differentiated patients from controls (27). The odds ratios were especially high in females subgroup (27). In the group of Polish children after stroke, *MTHFR* polymorphism seemed to be a risk factor for ischemic stroke, but in opposite to the results in CAD patients, in the male subgroup (28).

The present study has some limitations: the largest one is low number of analyzed subjects and lack of measurements of HCys and folate levels. Studies based on larger group of patients and controls are needed to confirm obtained results. Another limitation of our study is the fact that part of the included CAD patients were undergoing a treatment with cholesterol-lowering drugs which reflected in serum lipid levels. Important issue is also selective control group.

In conclusion, present work showed that the 677C>T polymorphism within *MTHFR* gene is associated with the premature CAD only in a presence of given environmental risk factors, which is an interesting contribution to gene-environment interaction problem that may have clinical implications in the future.

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