Are the T/C Polymorphism of the *CYP17* Gene and the Tetranucleotide Repeat (TTTA) Polymorphism of the *CYP19* Gene Genetic Markers for Premature Coronary Artery Disease in Caucasians?

(T/C polymorphism of the *CYP17* gene / tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene / genetic marker / coronary artery disease/association study)

M. LETONJA[1], B. PETERLIN[2], D. BREGAR[3], D. PETROVIČ[3]

[1]Department of Internal Medicine, General Hospital of Ptuj, Ptuj, Slovenia

[2]Division of Medical Genetics, Department of Obstetrics and Gynecology, University Medical Centre, Ljubljana, Slovenia

[3]Institute of Histology and Embryology, Medical Faculty, University of Ljubljana, Ljubljana, Slovenia

Abstract. Gender differences in CAD have been clearly documented, and sex hormones have been recognized to influence the risk of CAD. The cytochrome P450c17α gene (*CYP17*) and the *CYP19* gene influence concentrations of sex hormones. In this cross-sectional association study we tested the hypothesis whether the T/C polymorphism of the *CYP17* gene and the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene are genetic markers for CAD in Caucasians. The TT genotype of the *CYP17* gene polymorphism was not associated with premature CAD in men and women combined (OR 0.9; 95% CI = 0.6–1.4; P = 0.7), in men only (OR 1; 95% CI = 0.6–1.8; P = 0.7), and in women only (OR 0.8; 95% CI = 0.5–1.4; P = 0.4). The tetranucleotide repeat (TTTA) *CYP19* gene polymorphism was not associated with premature CAD. Moreover, the genotypes containing the longer alleles (A6 or A7) were not associated with a lower incidence of CAD, and the genotypes containing the shorter alleles (A1 or A2) were not over-represented in the CAD patients. We may conclude that in Caucasian subjects neither the T/C *CYP17* gene polymorphism nor the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene contributes to the genetic susceptibility to CAD, therefore they may not be used as genetic markers for CAD risk assessment.

Gender differences in coronary artery disease (CAD) have been clearly documented, and sex hormones have been recognized to influence the risk of developing cardiovascular disease (Barrett-Conner et al., 1991; Poglajen et al., 2004). Several genes are involved in the synthesis of sex hormones; however, two genes that encode the cytochrome P450c17α and cytochrome P450 aromatase (P450aro) enzymes are mainly involved in the final production of sex steroids (Nakajin et al., 1981; Miller, 1988; Miller, 1996).

The cytochrome P450c17α gene (*CYP17*) encodes the cytochrome P450c17α enzyme, which is involved in the formation of precursors of testosterone and oestradiol. The T/C polymorphism of the *CYP17* gene creates a recognition site for the *Msp*A1 restriction enzyme and has been used to designate two alleles, A1 (T) and A2 (C). Subjects with the C allele have an additional Sp 1 site, which results in an increased expression of the gene (Kadonaga et al., 1986); in these subjects elevated plasma levels of some steroid hormones have been reported (Feigelson et al., 1998; Haiman et al., 1999).

The *CYP19* gene encodes the enzyme aromatase (P450aro) that is crucially involved in the production of oestrogens from androgens. In men and postmenopausal women the enzyme aromatase is the main source of circulating oestrogens produced mainly peripherally from androgenic steroids, whereas in premenopausal women the ovary is the main source of circulating oestradiol (Sasano et al., 1998). A tetranucleotide repeat polymorphism $[TTTA]_n$ located in intron 4 of the *CYP19* gene influences oestradiol and testosterone concentrations, i.e. subjects with a higher number of intronic tetranucleotide (TTTA) repeats have higher oestradiol and testosterone concentrations in comparison with subjects with a lower number of repeats (Berstein et al., 2001).

Associations of the *CYP17* and *CYP19* polymorphisms are being extensively studied to clarify their role in hormone-related cancers (breast, endometrial, prostate and ovarian cancer), osteoporosis, and hip frac-

Received February 18, 2005. Accepted April 29, 2005.

Corresponding author: Daniel Petrovič, Institute of Histology and Embryology, Medical Faculty of Ljubljana, Korytkova 2, SI-1105 Ljubljana, Slovenia. Tel.: (+386) 1 543 7367; Fax: (+386) 1 543 7361; e-mail: daniel.petrovic@mf.uni-lj.si

Abbreviation: CAD – coronary artery disease, HDL – high density lipoproteins, LDL – low density lipoproteins, MI – myocardial infarction.

tures (Bulun et al., 1994; Carani et al., 1997; Feigelson et al., 1997, 1998; Lea et al., 1997; Sasano et al., 1998; Haiman et al., 1999; Wadelius et al., 1999; Healey et al., 2000; Berstein et al., 2001; Haiman et al., 2001), but there are no reports available on the *CYP17* and *CYP19* polymorphisms and their relationship to atherosclerosis. We hypothesized that variability in genes (*CYP17*, *CYP19*) that control the hormone (oestrogens, testosterone) biosynthesis might affect the risk of CAD.

In this cross-sectional association study we tested the hypothesis whether the T/C polymorphism of the *CYP17* gene and the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene are genetic markers for CAD in Caucasians. Additionally, we tested the hypothesis whether the T/C polymorphism of the *CYP17* gene and the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene affect lipid metabolism in the same population.

Subjects and Methods

Subjects

In this cross-sectional case-control association study we enrolled 538 subjects: 337 patients (210 men and 127 women) with premature CAD (251 with myocardial infarction and 86 with unstable angina confirmed by coronary angiography) in the study group and 201 subjects in the control group (100 men and 101 women). Male patients with CAD were younger than 55 years, and female patients with CAD were younger than 65 years. The diagnosis of myocardial infarction (MI) was made according to the criteria of the World Health Organization. In 86 patients with unstable angina, the diagnosis of CAD was confirmed by coronary angiography: they had severe stenosis on coronary angiography (≥ 70% stenosis of at least one coronary artery or ≥ 50% stenosis of the left main coronary artery). The patients and control subjects came from independent families. The data and blood samples of age-matched controls were obtained from general practitioners. The controls did not have a history of angina pectoris or MI, and had normal electrocardiogram. All the subjects enrolled in the study were Slovenian. The research protocol was approved by the national medical ethics committee. After informed consent had been obtained from the patients and control subjects, a detailed interview was made. The physician interviewed each patient about the coronary risk factors (diabetes, cigarette smoking, arterial hypertension, body weight and height). Smoking habit was defined as a daily intake of more than 5 cigarettes. Arterial hypertension and diabetes mellitus were defined as binary variables and their respective diagnosis was made according to the World Health Organization criteria.

Lipid analysis

Total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) and triglycerides were determined by standard biochemical methods.

Genetic analysis

Blood samples were obtained from peripheral veins of the patients after informed consent. DNA was purified from buffy coats using a rapid DNA preparation method. The *CYP17* assay has been described previously (Kuligana et al., 2000). To achieve an efficient amplification from partially degraded archival DNA, only the short fragment of *CYP17* was subjected to the analysis. Use of the sense 5'-AAGGTGAAGATCAGGGTAG-3' (nucleotides 1856-1875) and antisense 5'-GCTAGGGTAAGCAGCAAGAG-3' (nucleotides 1981-2000) primers resulted in the appearance of the 145 bp fragment. The digestion of the PCR product by the restriction endonuclease *Msp*A1 allowed discrimination between the alleles A1 and A2 of the *CYP17* polymorphism: the size of the A1-specific fragment remained intact, whereas the A2 allele was digested for 75 and 70 bp sequences. PCR reactions were carried out in 10-μl aliquots containing about 50 ng of genomic DNA, 0.2 μM of each primer, 1 time reaction buffer, 0.2 mM dNTP, and 0.5 unit of Taq polymerase (Pharmacia). PCR reaction: 35 cycles with denaturation at 94°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C. An initial denaturation step of 1 min at 94°C and a final extension at 72°C for 20 s were used. The PCR products were digested for 3 h at 37°C using *Msp*A1, separated by agarose gel electrophoresis, and stained with ethidium bromide to identify the bp change.

For *CYP19* PCR genotyping as described before (Bernstein et al., 2001) we used the following primers: sense 5'-GGTACTTAGTTAGCTACAATC-3' (nucleotides 610-630) and antisense 5'-GGGTGATAGAGTCAGAGCCT-3' (nucleotides 721-740). PCR reactions were carried out in 10-μl aliquots containing about 1 μl of genomic DNA, 1 μM of each primer, 1 time PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, Triton X-100, and 0.5 unit heat-activated MDP-1 thermostable polymerase. PCR reaction: 30 cycles with denaturation at 95 °C for 35 s, annealing at 72 °C for 60 s, and 60 s synthesis. The PCR products were separated in 15% polyacrylamide nondenaturating gel, and visualized with ethidium bromide. This approach allowed the separation of seven different sizes of the alleles.

Statistical methods

Differences in mean values between CAD patients and controls were analysed by Student t-test and presented as means ± standard deviation (SD). Chi-square test was used to compare discrete variables. An odds ratio (OR) was calculated as a measure of relative risk.

Univariate analysis was used in the assessment of the effect of potential risk genotypes on the risk of CAD. Statistical analyses were performed using the SPSS program for Windows 98 version 12 (SPSS Inc., Chicago, IL).

Results

The genotype distribution of the T/C *CYP17* gene polymorphism in the CAD group and in the control group were compatible with Hardy-Weinberg expectations (CAD group: $\chi^2 = 0.15$, P = 0.69; control group: $\chi^2 = 0.50$, P = 0.48) (Table 1).

The T/C *CYP17* gene polymorphism was not associated with premature CAD in men and women combined, in men alone, and in women alone, respectively (Table 1). The tetranucleotide repeat (TTTA) *CYP19* gene polymorphism was not associated with premature CAD. Moreover, the genotypes containing the longer alleles (A6 or A7) were not associated with a lower incidence of CAD, and the genotypes containing the shorter alleles (A1/A1, A1/A2, A2/A2) were not overrepresented in the CAD patients (Table 2).

As for lipid metabolism, we did not find statistically significant differences in lipid parameters (total choles-

Table 1. Distribution of the genotypes of the T/C CYP 17 gene polymorphism ($\chi^2 = 0.6$, P = 0.7) among CAD patients and controls

Variable	CAD patients (%)	Controls (%)	OR (95% CI)1	P
Genotypes – men and women combined				
genotype CC (A2/A2)	62 (18.4 %)	41 (20.4%)	0.9 (0.6-1.4)[2]	0.7[2]
genotype CT (A1/A2)	170 (50.4 %)	94 (46.8%)		
genotype TT (A1/A1)	105 (31.2 %)	66 (32.8%)	0.9 (0.6-1.3)[3]	0.6[3]
	337	201		
Alleles				
C (A2)	294 (43.6 %)	89 (43.8%)		0.9[4]
T (A1)	380 (56.4 %)	113 (56.2%)		
	674	402		
Genotypes – men only				
genotype CC (A2/A2)	38 (18.1 %)	17 (17.0%)	1.1 (0.6-2.2)[2]	0.7[2]
genotype CT (A1/A2)	107 (50.9 %)	53 (53.0%)		
genotype TT (A1/A1)	65 (31.0 %)	30 (30.0%)	1.0 (0.6-1.8)[3]	0.9[3]
	210	100		
Alleles				
C (A2)	183 (43.6 %)	87 (43.5%)		1.0[4]
T (A1)	237 (56.4 %)	113 (56.7%)		
	420	200		
Genotypes – women only				
genotype CC (A2/A2)	24 (18.9 %)	24 (23.8%)	0.8 (0.4-1.4)[2]	0.4[2]
genotype CT (A1/A2)	63 (49.6 %)	41 (40.6%)		
genotype TT (A1/A1)	40 (31.5 %)	36 (35.6%)	0.8 (0.5-1.4)[3]	0.5[3]
	127	101		
Alleles				
C (A2)	111 (43.7 %)	89 (44.1%)		0.9[4]
T (A1)	143 (56.3 %)	113 (55.9%)		
	254	202		

[1]Odds ratio (95% confidence interval), [2]OR (95% CI) and P value for the recessive model (genotype CC vs. genotype TC plus genotype TT), [3]OR (95% CI) and P value for the recessive model (genotype TT vs. genotype TC plus genotype CC), [4]P value for allele distribution

Table 2. Distribution of CYP19 alleles/genotypes in the CAD group and in the control group

Alleles	Total group (Pearson $\chi^2 = 1.4$, P = 0.9)		Men (Pearson $\chi^2 = 8.2$, P = 0.2)		Women (Pearson $\chi^2 = 6.7$, P = 0.35)	
	CAD	**Control**	**CAD**	**Control**	**CAD**	**Control**
A1	221 (33.9)	112 (32.9)	143 (34.0)	59 (35.5)	78 (33.6)	53 (30.5)
A2	105 (16.1)	50 (14.7)	75 (17.9)	20 (12.0)	30 (12.9)	30 (17.2)
A3	54 (8.3)	31 (9.1)	38 (9.0)	18 (10.8)	16 (6.9)	13 (7.5)
A4	5 (0.8)	2 (0.6)	5 (1.2)	0	0	2 (1.1)
A5	15 (2.3)	6 (1.8)	11 (2.6)	5 (3.0)	4 (1.7)	1 (0.6)
A6	221 (33.9)	120 (35.3)	136 (32.4)	54 (32.5)	85 (36.6)	66 (37.9)
A7	31 (4.8)	19 (5.6)	12 (2.9)	10 (6.0)	19 (8.2)	9 (5.2)
total	**652**	**340**	**420**	**166**	**232**	**174**
	Total group (Pearson $\chi^2 =$ 13.1, P = 0.9)		**Men (Pearson $\chi^2 = 18.3$, P = 0.6)**		**Women (Pearson $\chi^2 = 18.9$, P = 0.40)**	
Genotypes	**CAD**	**Control**	**CAD**	**Control**	**CAD**	**Control**
	326	170	210	83	116	87
A1A1	44 (13.5)	23 (13.5)	26 (12.4)	15 (18.1)	18 (15.5)	8 (9.2)
A2A2	9 (2.8)	3 (1.8)	6 (2.9)	1 (1.2)	3 (2.6)	2 (2.3)
A3A3	6 (1.8)	2 (1.2)	5 (2.4)	2 (2.4)	1 (0.9)	0
A6A6	43 (13.2)	21 (12.4)	25 (11.9)	10 (12.0)	18 (15.5)	11 (12.6)
A7A7	6 (1.8)	4 (2.4)	1 (0.5)	2 (2.4)	5 (4.3)	2 (2.3)
A1A2	40 (12.3)	19 (11.2)	29 (13.8)	7 (8.4)	11 (9.5)	12 (13.8)
A1A3	11 (3.4)	5 (2.9)	8 (3.8)	4 (4.8)	3 (2.6)	1 (1.1)
A1A5	4 (1.2)	3 (1.8)	4 (1.9)	3 (3.6)	0	0
A1A6	70 (21.5)	36 (21.2)	44 (21.0)	13 (15.7)	26 (22.4)	23 (26.4)
A1A7	8 (2.5)	3 (1.8)	6 (2.9)	2 (2.4)	2 (1.7)	1 (1.1)
A2A3	4 (1.2)	2 (1.2)	4 (1.9)	1 (1.2)	0	1 (1.1)
A2A4	4 (1.2)	0	4 (1.9)	0	0	0
A2A5	3 (0.9)	1 (0.6)	1 (0.5)	1 (1.2)	2 (1.7)	0
A2A6	34 (10.4)	18 (10.6)	24 (11.4)	8 (9.6)	10 (8.6)	10 (11.5)
A2A7	2 (0.6)	4 (2.4)	1 (0.5)	1 (1.2)	1 (0.9)	3 (3.4)
A3A5	1 (0.3)	1 (0.6)	1 (0.5)	0	0	1 (1.1)
A3A6	21 (6.4)	18 (10.6)	13 (6.2)	9 (10.8)	8 (6.9)	9 (10.3)
A3A7	5 (1.5)	1 (0.6)	2 (1.0)	0	3 (2.6)	1 (1.1)
A4A6	1 (0.3)	2 (1.2)	1 (0.5)	0	0	2 (2.3)
A5A6	5 (1.5)	1 (0.6)	3 (1.4)	1 (1.2)	2 (1.7)	0
A6A7	4 (1.2)	3 (1.8)	1 (0.5)	3 (3.6)	3 (2.6)	0
All genotypes with A6	139 (42.6)	80 (47.1)	84 (40.0)	35 (42.2)	55 (47.4)	45 (51.7)
All genotypes with A7	25 (7.7)	15 (8.8)	11 (5.2)	8 (9.6)	14 (12.1)	7 (8.0)

terol, LDL cholesterol, HDL cholesterol and triglycerides) among the subjects (patients and controls) with regard to the genotypes of the T/C *CYP17* gene polymorphism (TT vs. TC, TT vs. CC, CC vs. TC; data not shown). Neither were the statistically significant differences found in lipid parameters in the female subjects (patients and controls; data not shown). On the other hand, the male subjects (patients and controls) with the TT genotype had statistically significantly higher total and LDL cholesterol levels than the male subjects

(patients and controls) with the genotype TC (total cholesterol: 6.59 ± 2.13 mmol/l vs. 6.09 ± 1.43 mmol/l, $P < 0.05$; LDL cholesterol: 4.29 ± 1.22 mmol/l vs. 4.08 ± 1.38 mmol/l, $P < 0.05$), whereas no statistically significant differences were found in other lipid parameters (data not shown).

Further, we did not find statistically significant differences in lipid parameters with regard to the different number of repeats (A1A1 plus A1A2 plus A2A2 vs. A6A6 plus A6A7 plus A7A7; data not shown).

In our study there were no statistically significant differences in the body mass index among the subjects (men and women combined, in men alone, and in women alone, respectively) with regard to different genotypes (TT vs. TC plus CC; A1A1 plus A1A2 plus A2A2 vs. A6A6 plus A6A7 plus A7A7; data not shown).

Discussion

In this cross-sectional case-control study we demonstrated that neither the T/C *CYP17* gene polymorphism nor the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene was associated with premature CAD.

Recently, women with the CC genotype were reported to have elevated levels of oestrone, oestradiol, testosterone, androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulphate (DHEAS) (Haiman et al., 1999), whereas such information is not available for men. Moreover, women with the TT genotype were associated with hyperinsulinaemia/increased insulin secretion (Berstein et al., 2002). Both pathways (hormone pathway and insulin pathway), however, may be important in the pathogenesis of CAD. In our study neither the CC genotype nor the TT genotype of the T/C *CYP17* gene polymorphism affected the risk of CAD in Caucasians regardless of sex. Moreover, neither the CC genotype nor the TT genotype was associated with either decreased or increased cholesterol levels in men and women combined. A subgroup analysis of men demonstrated statistically significant differences in total and LDL cholesterol levels between men with the TT genotype and men with the TC genotype ($P < 0.05$), i.e. men with the TT genotype had higher total and LDL cholesterol levels than men with the CT genotype. Moreover, we did not demonstrate that the T/C *CYP17* gene polymorphism significantly affected the body mass index.

In the study we did not find the difference in the allele/genotype distribution of the tetranucleotide repeat (TTTA) *CYP19* gene polymorphism between the patients with premature CAD (men and women combined, men only, women only) and the control subjects. Bearing in mind the report of increased oestradiol and testosterone concentrations in A6 and A7 carriers (Berstein et al., 2001), we expected a favourable effect of longer alleles (A6 and A7) on the risk of CAD. We did not, however, observe the carriers of the longer alleles (A6 and/or A7) of the *CYP19* gene polymorphism to have a decreased risk of CAD. Additionally, we did not find significant differences in lipid parameters between the subjects with shorter alleles (allele A1 and A2) and longer alleles (A6 and A7). Similarly, we did not demonstrate that the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene significantly affected the body mass index.

Our findings are in accordance with some previous studies that failed to demonstrate a correlation between the total levels of circulating oestrogens and the degree of atherosclerosis in men and post-menopausal women (Cauley et al., 1992; Phillips et al., 1994). Among others, oestrogens influence the progression of atherosclerosis by decreasing LDL cholesterol and increasing HDL cholesterol (Knopp 1998; Mendelson et al., 1999). A direct biological action of oestrogen on smooth muscle cells of the vessel wall may be the explanation for the attenuating effect of oestrogen on atherosclerosis. Human studies indicate that testosterone and other sex prohormones attenuate atherogenesis by being converted to oestrogen with enzyme aromatase expressed in the vessel wall, which has been proved in cultured smooth muscle cells of human arterial wall and normal aorta (Harada et al., 1999; Murakami et al., 2001).

We may conclude that in Caucasian subjects neither the T/C *CYP17* gene polymorphism nor the tetranucleotide repeat (TTTA) polymorphism of the *CYP19* gene contributes to the genetic susceptibility to CAD, therefore they may not be used as genetic markers for CAD risk assessment.

Acknowledgements

The authors thank Ms Mojca Pirc, BA, for revising the English.

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