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Analysis of Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 Gene K121Q Polymorphism Association with Some Risk Factors of Atherosclerosis in Patients with Acute Coronary Syndrome[1]

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Abstract—The present study was performed to investigate whether common single-nucleotide polymorphism K121Q (rs1044498) of the *ENPP1* gene is associated with the known risk factors of atherosclerosis (overweight, dyslipoproteinemia, hypertension, diabetes, smoking, and hypercoagulability) in persons with acute coronary syndrome. Venous blood of 118 patients was genotyped for the polymorphism by PCR and restriction fragment length polymorphism method. In patients divided into two subgroups according to their genotype (KK and KQ + QQ), the statistically significant differences were revealed only for plasma LDL-cholesterol level and fibrinolytic activity. The carriers of minor allele (KQ + QQ) had lower LDL-cholesterol concentration and the time of fibrinolysis than the major allele homozygotes (KK). The division of patients into subgroups according to presence or absence of some risk factors for atherosclerosis showed no statistically significant differences between K121Q genotype distributions for any of the comparison.

Keywords: ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), acute coronary syndrome, single nucleotide polymorphism, risk factors

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INTRODUCTION

The main cause of acute coronary syndrome (ACS) that encompasses a spectrum of unstable coronary artery disease from unstable angina to transmural myocardial infarction is the formation of thrombus on complicated atheromatous plaque. Both atherosclerosis and blood coagulation are the processes which depend not only on environmental risk factors but also on genetic features of organism. There is growing evidence that genetic factors, especially single nucleotide polymorphism (SNP) of a wide range of genes, play an important role in the development of atherosclerosis and its complications [1–3]. Among the genes, which products can be involved in progression of atherosclerotic lesions, the genes concerning arterial calcification are presently of great interest [4]. It is because the deposition of hydroxyapatite into atheromatous plaques makes them very unstable and facilitates the futher rupture and thrombus formation [5].

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is an enzyme with the extracellular catalytic domain that is able to cleave sugar-phosphate, phosphosulfate, pyrophosphate, and phosphodiesterase linkages [6]. The very important product of ENPP1 activity is inorganic pyrophosphate (PP_i) that is known to be one of the most potent inhibitor of arterial calcification [7–9]. The massive mineralization of arteries has been shown to develop when the activity of ENNP1 falls off and the formation of PP_i is fully disturbed. Generalized deposits of calcium salts in arterial tissues are the most impressive feature in genetically knocked out mice (*ENPP1* –/–) and in humans who have defective *ENNP1* gene [10].

There are several lines of evidence indicating the ENPP1 contribution to the decreased insulin receptor function [6]. Among those is the fact that one of the most studied SNP of *ENPP1* gene, known as K121Q, is related to insulin resistance (IR) and development of type 2 diabetes (T2D) [11, 12]. Some data suggest that the Q121 allele is associated with an increased risk of earlier onset of myocardial infarction [13, 14]. It seems likely that this association is secondary to the effect of the Q121 allele on IR and T2D, which, in turn, predispose to atherosclerosis. The question remains open whether K121Q polymorphism itself influence arteriosclerotic process and ACS directly, i.e. by its own enzymatic activity which is not related to insulin receptor inhibiting, or through the risk factors other than IR and T2D.

[1] The article was translated by the authors.

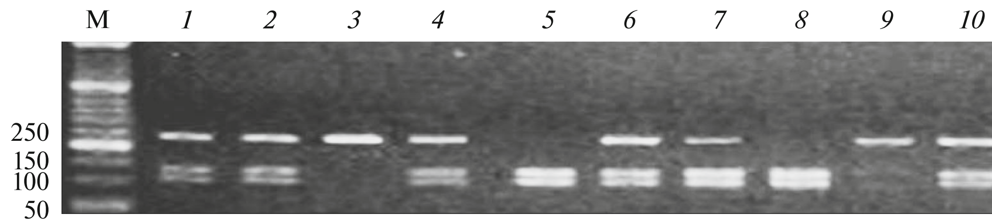

Fig. 1. Results of *ENPP1* K121Q polymorphism restriction analysis. M—molecular marker (bp—base pairs); lanes (*3*, *9*) KK-genotype; lanes (*1*, *2*, *4*, *6*, *7*, *10*) KQ-genotype; lanes (*5*, *8*) QQ-genotype.

The aim of the present study was to analyze the association of the *ENPP1* gene K121Q polymorphism with well-established risk factors of atherosclerosis such as hypercholesterolemia, hypertension, high body mass index, diabetes, smoking, and hypercoagulability in the patients with ACS presenting Ukrainian population.

MATERIAL AND METHODS

Subjects

The studied group included 118 ACS patients with a mean age of 55.9 ± 0.89 years admitted to Sumy Clinical Hospital no. 1. Diagnosis of ACS was established based on clinical, electrocardiography and biochemical examinations according to the recommendations of WHO experts and according to the recommendations of European and American cardiologic societies [15, 16]. Patients with hereditary and innate diseases, severe metabolic pathologies including a severe form of diabetes mellitus (proliferative stage of retinopathy, 2–5 stages of nephropathy, encephalopathy), marked renal and liver failures, deficiencies of the haemostatic system, oncology and systemic pathologies, chronic heart failure of II–III stage, true cardiogenic shock were excluded from the study group. The clinical signs included generally accepted parameters related to the risk factors for atherosclerosis and ACS: body mass index (BMI), blood pressure (BP), the content of lipids and lipoproteins in blood plasma, and some indices of blood coagulation (prothrombin time). According to these parameters, all patients were divided into the pairs of subgroups defined by (1) BMI (<25 kg/m^2 or ≥ 25 kg/m^2), (2) BP (non-hypertensive or hypertensive: systolic BP > 140 mmHg, diastolic BP > 90 mmHg), (3) low density lipoprotein cholesterol (LDL-C) concentration (≤3.5 mmol/L or >3.5 mmol/L), (4) diabetes mellitus type 2 (non-diabetic or diabetic), (5) smoking (non-smoking or smoking), and (6) prothrombin time (≥9 or <9″).

The study had been previously approved by the Ethic Committee on Medical Research of the Medical Institute of Sumy State University. An appropriate informed consent was obtained from all patients. Blood sampling for genotyping was performed under sterile conditions into 2.7 mL S-Monovette (Sarstedt, Germany) containing EDTA potassium salt as an anticoagulant, samples were frozen and stored at –20°C.

Amplification and Genotyping

DNA for genotyping was extracted from the venous blood using commercially available kits (Isogene Lab Ltd, Russia) according to the manufacturer's protocol. To identify *ENPPI* K121Q polymorphism (rs1044498) the polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis was performed. Specific region of the *ENPP1* gene was amplified using a pair of specific primers: upstream (sense)–5'-CTGTGTTCACTTTGGACATGTTG-3' and downstream (antisense)–5'-GACGCTGGAAGATACCAGGCTG-3'. Primers were provided by Metabion (Germany). PCR was performed for 33 cycles in a 25 μL volume containing 50–100 ng of DNA, 5 μL 5X PCR-buffer, 1.5 mM/L magnesium sulfate, 200 μM/L of each dNTP, 15 pM/L of each primer and 0.75U of *Taq*DNA polymerase (Thermo Scientific, USA). PCR was carried out in a termocycler GeneAmpPCR System 2700 (Applied Biosystems, USA). Six microlitres (6 μL) of the PCR products (238 bp) were subjected to digestion with 5U *Eco*47I (*Ava*II) (Thermo Scientific, USA) and incubated at 37°C for 18 h. When cytosine is at the position 48 213 of the exon 4, *Eco*47I restriction enzyme produces two fragments of 148 and 90 bp in length. Substitution of cytosine for adenine prevents restriction and the amplified fragment of the exon 4 (238 bp) cannot be cleaved (Fig. 1). The restriction fragments were separated by electrophoresis and analysed on an ethidium bromide-stained 2.5% agarose gel visualized using ultraviolet transillumination.

Statistical Analysis

To test if the K121Q genotypes distribution followed Hardy-Weinberg equilibrium as well as for the comparison of the K121Q allele and genotype frequencies between different study subgroups χ^2-test was used. Since there were few individuals with the QQ genotype, we tested whether variable means differed significantly between subjects with and without the Q variant (KQ + QQ vs. KK). A comparison of

Table 1. Clinical characteristics of acute coronary syndrome patients with different *ENPP1* gene K121Q genotype

Parameter	KK	KQ + QQ	Total	*P*
n	79	39	118	
Age, years	56.1 ± 1.17	55.5 ± 1.25	55.9 ± 0.89	0.699
Gender, M/F	62/17	30/9	92/26	0.848
BMI (M), kg/m^2	28.0 ± 0.54	26.9 ± 0.6	27.6 ± 0.42	0.593
BMI (F), kg/m^2	31.0 ± 1.11	32.4 ± 1.55	31.5 ± 0.9	0.467
Systolic BP, mmHg	140 ± 1.9	142 ± 3.3	141 ± 1.7	0.560
Diastolic BP, mmHg	89 ± 1.1	90 ± 1.7	89 ± 0.9	0.627
Total cholesterol, mmol/L	6.6 ± 0.16	6.0 ± 0.24	6.4 ± 0.13	0.075
LDL-cholesterol, mmol/L	4.7 ± 0.17	4.1 ± 0.26	4.5 ± 0.14	0.039
HDL-cholesterol, mmol/L	1.0 ± 0.02	1.1 ± 0.04	1.0 ± 0.02	0.062
Triglyceride, mmol/L	1.7 ± 0.1	1.8 ± 0.14	1.8 ± 0.08	0.598
Prothrombin time, s	10.4 ± 0.18	11.0 ± 0.31	10.6 ± 0.16	0.112
Thrombin time, s	17.3 ± 0.48	18.8 ± 0.8	17.8 ± 0.42	0.077
Fibrinolytic activity, s	479 ± 4.1	462 ± 6.3	473 ± 3.5	0.022
Fasting glucose, mmol/L	8.0 ± 0.32	7.8 ± 0.37	7.9 ± 0.24	0.688
Diabetes, %	23 (29.1)	7 (17.9)	30 (25.4)	0.190
Smoking, %	34 (43.0)	20 (51.3)	54 (45.8)	0.398

Data are mean ± SE; *n*—number of subjects; HDL—igh density lipoprotein; LDL—low density lipoprotein.

variables between the groups of genotypes was performed using two-tailed Student's *t*-test. Differences were considered statistically significant with a *P*-value <0.05. All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 17.0, SPSS Inc, Chicago, IL).

RESULTS

Results of the *ENPP1* K121Q genotyping among patients with ACS showed that 79 (66.9%) subjects had KK genotype, 36 (30.5%)—KQ genotype, and 3 (2.5%) individuals were minor allele homozygotes (QQ). Herewith the genotype distributions of *ENPP1* K121Q locus in case group (minor allele frequency—0.181) were consistent with the Hardy-Weinberg equilibrium ($p > 0.05$).

The known ACS risk factors include increased BMI, hypertension, elevated levels of cholesterol and LDL in blood plasma, diabetes mellitus, smoking, and hypercoagulability, i.e. factors relating to atherogenesis and thrombi formation. In Table 1, some clinical characteristics manifesting the abovementioned ACS risk factors in the patients divided into two subgroups according to their genotype (KK and KQ + QQ) are presented.

The statistically significant differences were revealed between these two subgroups only for LDL-cholesterol level and fibrinolytic activity. The carriers of minor allele (KQ + QQ) had lower LDL-cholesterol concentration and the time of fibrinolysis than the major allele homozygotes (KK).

The division of patients into subgroups according to the presence or absence of known ACS risk factors allowed the comparative analysis of the K121Q genotypes distribution. As shown in Table 2, statistically significant differences were not established for any of the comparison. The similar analysis in subjects of different sex also did not show any significant difference in the *ENPP1* K121Q genotype frequencies (data are not shown).

DISCUSSION

Two important features of ENPP1 seem to be involved in the development of arteriosclerotic lesions and their complications. First of them is the ability of ENPP1 to generate PP_i, a major physiologic inhibitor of calcification that exerts its effects by inhibiting hydroxyapatite crystal growth [9, 17]. Arterial wall calcification is a common pathological process that is of great significance by itself (Mönckeberg's sclerosis) and can complicate atherosclerotic plaques contributing to their instability.

The second feature of ENPP1 concerns the insulin resistance and is reported not to be related to enzyme activity of this protein and PP_i production. It is shown that catalytic domain of ENNP1 is not involved in inhibiting the insulin receptors because the mutation inactivating the enzymatic activity of *ENPP1* does not impair its ability to inhibit function of insulin receptors [18].

Table 2. Distribution of genotypes by the ENPP1 gene K121Q polymorphism in subgroups of ACS patients with regards to different disease risk factors

Genotype	BMI, *n*, %		Blood pressure, *n*, %	
	<25 kg/m^2	≥25 kg/m^2	normal	elevated
KK	11 (52.4)	68 (70.1)	30 (65.2)	49 (68.1)
KQ + QQ	10 (47.6)	29 (29.9)	16 (34.8)	23 (31.9)
In total	21	97	46	72
	$P = 0.118$		$P = 0.749$	
Genotype	**Diabetes, *n*, %**		**Smoking, *n*, %**	
	non-diabetic	diabetic	non-smokers	smokers
KK	56 (63.3)	23 (76.7)	45 (70.3)	34 (63.0)
KQ + QQ	32 (36.4)	7 (23.3)	19 (29.7)	20 (37.0)
In total	88	30	64	54
	$P = 0.190$		$P = 0.398$	
Genotype	**Blood plasma lipoproteins, *n*, %**		**Blood coagulation, *n*, %**	
	AI ≤ 3	AI > 3	PT ≥ 9 s	PT < 9 s
KK	11 (55.0)	68 (69.4)	64 (66.7)	15 (68.2)
KQ + QQ	9 (45.0)	30 (30.6)	32 (33.3)	7 (31.8)
In total	20	98	96	22
	$P = 0.213$		$P = 0.892$	

n—Number of subjects, BMI—body mass index, AI—atherogenicity index, PT—prothrombin time.

However, whether inhibition of insulin receptor signaling by ENPP1 is dependent upon pyrophosphatase/phosphodiesterase its activity remains controversial. Thus, Chin et al. [19] studying the extracellular domain of the human ENPP1 in its native form or with some mutations have demonstrated the functional dependency between two features of ENPP1.

As shown in many clinical studies, both increased expression and genetic polymorphism of *ENPP1*, especially K121Q, are associated with IR and T2D in humans [11, 20]. A wide range of case-control studies have been carried out to asses the possible association between the *ENPP1* gene K121Q polymorphism and IR, T2D, and overweight/obesity in many Caucasian and not-Caucasian populations [6]. Although the published results are rather contradictory, it can be concluded that in many cases there are positive associations between Q variant of *ENPP1* gene and aforementioned pathological conditions. All of these conditions are considered tightly pathogenetic connected with atherosclerotic process, which is the main cause of ACS. There is evidence suggesting that the Q allele is associated with an increased risk of earlier onset of myocardial infarction [13, 14]. This association may be secondary to the effect of the Q allele on IR, T2D, and overweight/obesity that all predispose to atherosclerosis.

In the present study, we explored associations between genetic variation in the *ENPP1* gene and some risk factors of atherosclerosis in patients with ACS. Analyzing the K121Q genotypes distribution in various subgroups of patients, we did not find any differences in this parameter between subjects with and without well-known risk factors for ACS. Only two indices, LDL-cholesterol concentration and fibrinolytic activity, were significantly different in KK-homozygotes and carriers of Q-allele. Both of these parameters were lower in patients with Q-variant of *ENPP1* gene than in subjects with KK-genotype. However, it is difficult to say whether these differences are of any importance for pathogenesis of ACS.

CONCLUSIONS

In the present study, it was shown no differences in *ENPP1* gene K121Q genotypes distribution in the patients with ACS divided into subgroups according to the presence or absence of known risk factors for atherosclerosis: overweight, dyslipoproteinemia, hypertension, diabetes, smoking, and hypercoagulability. Only two parameters, plasma LDL-cholesterol concentration and fibrinolytic activity were significantly lower in patients with Q-variant of *ENPP1* gene than in subjects with KK-genotype.

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