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Impact of smoking status on particular genetic polymorphisms associations with cardiovascular diseases

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Abstract

Background. Genes encoding : angiotensin converting enzyme (ACE), platelet glycoprotein IIb/IIIa (GP IIb/IIIa) and endothelial nitric oxide synthase (eNOS) meet the criteria for a candidate gene for cardiovascular disease, including myocardial infarction(MI). Myocardial infarction results from an interplay of both genetic and environmental factors. Certain genetic polymorphisms seem to modify the deletorious impact of environmental factors, such as cigarette smoking, and could modify the inherited risk. The aim of the presented study was to assess the influence of cigarette smoking on the incidence of myocardial infarction in the presence of particular ACE, GPIIb/IIIa and eNOS genes polymorphic variants and genotypes.

Material and methods. 166 individuals of Polish origin were genotyped, all of whom suffered from MI, or had survived it – 138 smokers and 28 non-smokers, all under 55 years of age. Polymorphisms were detected using PCR-RLFP method.

Results. Genotypes including I allele of ACE gene I/D polymorphism (II and ID) occurred more frequently in smokers with MI compared to non-smokers (p=0.013). Similar findings were observed with T allele of the eNOS G894T polymorphism, and occurred more frequently among smokers when compared to non-smokers (p=0.039). The frequencies of A2 variant of the GPIIb/IIIa PL A1/A2 polymorphism were similar in smoking and non-smoking myocardial interaction patients.

Conclusions. Cigarette smoking seems to have an impact on the associations between myocardial infarction with ACE gene I/D and eNOS gene G894T polymorphisms, but no interaction was observed with GPIIb/IIIa gene PLA1/A2 plymorphism.

Key words

myocardial infarction, tobacco smoking, genetic polymorphism, angiotensin converting enzyme gene, platelet glycoprotein IIb/IIIa gene, endothelial nitric oxide synthase gene.

INTRODUCTION

Both genetic and environmental factors play important roles in the pathogenesis of coronary artery disease. Myocardial infarction (MI) is a common cause of premature deaths in people under 55 years of age. Therefore, myocardial infarction is a life-threatening condition, and increasing data suggests that there is an interplay between environmental factors and genetic predisposition, and factors such as cigarette smoking could have a potential role in modifying gene expression. Cigarette smoke is a complex medium containing about 4,000 different components, e.g. carbon monoxide, carbon dioxide, ammonia, hydrogen cyanide, nitrogen oxides, and nicotine. Cigarette smoking is a potent factor causing oxidative stress and decreasing nitric oxide synthesis that leads to endothelial dysfunction, with clinical consequences and MI among them [1].

Regarding the important role of angiotensin-converting enzyme (ACE) in physiology and pathology, the gene coding this protein meets criteria for a candidate gene for cardiovascular disease, including myocardial infarction. ACE gene was cloned and mapped to the long arm of chromosome 17 (17q23). The insertion/deletion (I/D) polymorphism of a 287 base pair sequence, located in intron 16 has been extensively studied over the past decade [2]. This polymorphism has a major effect on plasma and cellular enzyme activity. DD genotype is associated with about 50% higher plasma activity of the enzyme [3]. It was hypothesised that insertion variant (I allele) contains a so-called silencer sequence that causes a decrease in ACE gene expression.

MI often results from the development of an acute occlusive thrombus in an atherosclerotic coronary artery. Platelets have a key role in this process, particularly platelet membrane glycoprotein IIb/IIIa, which plays a key role in the platelet aggregation. A polymorphism of the gene encoding the IIIa subunit, with the two allele forms PLA1 and PLA2, has been identified. The PLA1/A2 polymorphism is a T to C substitution at position 1565 in exon 2 of the glycoprotein IIIa gene. Some studies suggest that the PLA2 allele confers an increased risk for myocardial infarction [4].

Ichiki et al. proved that long-term smoking significantly impaired platelet-derived NO release [1]. Human endothelial nitric synthase (eNOS) gene polymorphism is under intense investigation as a risk factor for MI. The endothelium-derived nitric oxide (NO) is synthesized from L-arginine by eNOS and plays an important role in regulating functions in the cardiovascular system, including vasorelaxation and inhibition of platelet aggregation. Biochemical or physical injury to the endothelium impairs production or function of endothelium-derived vasoprotective mediators of vascular, such as nitric oxide. Lack of NO results in increased vascular

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contractions to vasoconstrictors, such as endothelin-1, thromboxanes and serotonin. Defects in endothelial NO function is associated with cardiovascular risk factors and plays a major role in progressive of atherosclerotic disease. Human eNOS is encoded by the gene mapped on chromosome 7 [5]. Polymorphism of eNOS gene (Glu298Asp – the G894T substitution within exon 7) is the most frequently studied, and has been reported to be in association with MI in young patients [6, 7, 8]. Wang XL et al. observed in current and ex-cigarette smokers, but not non-smokers, that there is a significant excess of homozygotes for the rare ecNOS4a allele in patients with a history of myocardial infarction [9].

The aim of the presented study was to assess the influence of cigarette smoking on the incidence of myocardial infarction in the presence of certain ACE, GP IIb/IIIa and eNOS gene polymorphic variants and genotypes.

MATERIAL AND METHODS

166 male individuals of Polish origin were genotyped, all of whom had suffered from acute myocardial infarction while were under the age of 55 years and had survived. In this group, 125 were current tobacco smokers, 13 reported smoking cessation, and 28 were non-smokers. The mean age of the subjects was 53.2 (SD ± 4,2) years. All subjects were hospitalised in the Department of Internal Medicine at the Medical University in Lublin. The study protocol was approved by the local Bioethics Committee (KE-0254/135/98).

After obtaining an informed consent from each individual, 15 ml of venous blood were drawn into an EDTA tube. The standard procedure for genomic DNA preparation from peripheral blood leukocytes was used [10].

To detect ACE gene I/D polymorphism a method of Rigat was used, with some modifications [3]. Polymerase chain reaction (PCR) with specific primers (sense: 5'-GAT GTG GCC ATC ACA TTC GTC AGA-3' and antisense: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3') was performed in 50 µl volume. The reaction mixture contained 500 ng DNA, 1 unit of Taq polymerase in standard buffer with the addition of 220 pM of each primer, 2.5 mM of each deoxynucleotide (dATP, dGPT, dCTP, dTTP), 1.5 mM $MgCl_2$ (all reagents from MBI Fermentas) and 1.5% dimethylsulfoxide (DMSO). DNA amplification was performed in PTC-200 thermocycler MJ Research. After initial denaturation at 94 °C for 6 min., 30 cycles followed consisting of denaturation at 94 °C for 1 min., annealing at 60 °C for 1 min., and chain elongation at 72 °C for 1 min. Final extension was at 72 °C for 7 mins. In the presence of insertion (allele I), 490bp band was seen, while 190bp band appeared in the case of deletion (allele D). To avoid mistyping of DD genotype, a second run PCR with insertion specific primers (sense: 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' and antisense: 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3') was performed by the method described by Odawara [11].

For the detection of GPIIb/IIIa gene polymorphism (PlA1/A2), polymerase chain reaction (PCR) with specific primers was used (sense: 5'-TTC TGA TTG CTG GAC TTC TCT T-3' and antisense: 5'-TCT CTC CCC ATG GCA AAG AGT-3') was performed in 50 µl volume. The reaction mixture contained 1 mg DNA, 1 unit of Taq polymerase, 50 mmol KCl, 10 mmol TRIS-HCl, 0.1 µmol of each primer, 200 µmol of each deoxynucleotide (dATP, dGPT, dCTP, dTTP), 1.5 mmol $MgCl_2$ (all reagents from MBI Fermentas). DNA amplification was performed in PTC-200 thermocycler MJ Research. After initial denaturation at 95 °C for 5 min., 34 cycles followed, consisting of denaturation at 95 °C for 1 min., annealing at 55 °C for 1 min., and chain elongation at 72 °C for 2 min. Final extension was at 72 °C for 7 mins. MspI restriction fragment length polymorphism (RFLP) was then performed.

To detect eNOS gene polymorphism, polymerase chain reaction (PCR) with specific primers was used (sense: 5'-AAG GCA GGA GAC AGT GGA TGA A-3' and antisense: 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3') was performed in 50 µl volume. The reaction mixture contained 500 ng DNA, 1 unit of Taq polymerase, 50 mmol KCl, 10 mmol TRIS-HCl, 0.1 µmol of each primer, 200 µmol of each deoxynucleotide (dATP, dGPT, dCTP, dTTP), 1.5 mmol $MgCl_2$ (all reagents from MBI Fermentas). DNA amplification was performed in PTC-200 thermocycler MJ Research. After initial denaturation at 95 °C for 7 min., 34 cycles followed, consisting of denaturation at 94 °C for 1 min., annealing at 65 °C for 1 min., and chain elongation at 72 °C for 2 min. Final extension was at 72 °C for 7 min. Ban II restriction fragment length polymorphism (RFLP) was then performed. Products of PCR and PCR-RFLP were electrophoresed on 2% agarose gel (Prona) and visualised by ultraviolet transillumination after ethidium bromide staining.

Statistical analysis. Differences in allele frequencies and genotyped distribution between cases and controls were analysed by Pearson χ^2, and statistic with different degrees of freedom by using Yate's correction in the case of comparisons of small groups. The p values less than 0.05 were considered significant.

RESULTS

Polymorphic alleles frequencies and genotype distribution of the ACE gene I/D polymorphism are shown in Table 1. Polymorphic variants (I and D) frequencies did not differ between groups depending on smoking status. However, genotype containing I allele (II + ID), thought to be associated with a lower risk for coronary artery disease, occurred more frequently among smokers with myocardial infarction than in the non-smoking group. The difference reached the statistical significance level.

Table 1. Polymorphic allele frequencies and genotype distribution of the ACE gene I/D polymorphism in smoker and non-smoker groups of myocardial infarction male subjects

Myocardial infarction patients	No. of subjects	No. of alleles	Alleles		Genotypes	
			I	D	DD	ID + II
Smokers with MI	138	276	0.48	0.52	0.19	0.81
Non-smokers with MI	28	56	0.35	0.65	0.38	0.62
			p>0.05		p=0.013	

Polymorphic alleles frequencies and genotype distribution of the GP IIb/IIIa gene PLA1/A2 polymorphism are shown in Table 2. Because there were only a few A2A2 genotype bearers in the study, group genotypes containing A2 variant (A1A2 and A2A2) were analysed together.

The obtained results revealed similar frequencies of polymorphic alleles in both groups. Genotype distribution

Table 2. Polymorphic allele frequencies and genotype distribution of the GPIIb/IIIa gene PLA1/A2 polymorphism in smoker and non-smoker myocardial infarction subjects

Myocardial infarction patients	No. of subjects	No. of alleles	Alleles		Genotypes	
			A1	A2	A1A1	A1A2 + A2A2
Smokers with MI	138	276	0.81	0.19	0.67	0.33
Non-smokers with MI	28	56	0.73	0.27	0.54	0.46
			p>0.05		p>0.05	

did not differ substantially between the smoking and non-smoking group of patients.

Polymorphic alleles frequencies and genotypes distribution of the eNOS gene G894T polymorphism are shown in Table 3. Polymorphic allele frequencies were similar in both groups; however, genotypes containing T allele (TT+GT) occurred more frequently among smokers suffering from myocardial infarction. The difference was statistically significant (p=0.039).

Table 3. Polymorphic allele frequencies and genotype distribution of the eNOS gene G489T polymorphism in smoker and non-smoker subjects with myocardial infarction

Myocardial infarction patients	No. of subjects	No. of alleles	Alleles		Genotypes	
			G	T	GG	TT+TG
Smokers with MI	138	276	0.86	0.14	0.71	0.29
Non-smokers with MI	28	56	0.92	0.08	0.86	0.14
			p>0.05		p=0.039	

DISCUSSION

It was found that patients with genotype II or ID, who were thought to be at lower risk for cardiovascular diseases than those with genotype DD, occurred more frequently in smokers when compared to non-smokers. The difference was statistically significant (p=0.013). A large population-based study performed by Arias-Vásquez et al. in a group of 6,968 elderly people showed an increased risk of total mortality in subjects who died below the age of 65 who carried the DD genotype [12]. This association was significant, the risk was restricted only to those who smoked. Arias-Vásquez et al. suggest that the individuals who carry the DD genotype appear to be susceptible to early mortality if they smoke, suggesting the possible interaction between smoking habit and this genotype [12]. Sayed-Tabatabaei et al. in the Rotterdam Study, determined the ACE I/D polymorphism and recorded the smoking status in a group of 6,714 participants [13]. Fatal and non-fatal myocardial infarction and mortality events were also recorded. There were no significant differences between the genotypes with regard MI to incidence. However, among the smokers, there was an increased risk of cardiovascular disease and mortality in bearers of the DD genotype when compared to the II genotype. The statistical analysis suggested that the ACE I/D polymorphism is not a strong risk factor for myocardial infarction but its interaction with smoking might play a certain role in cardiovascular mortality, especially at younger age [13]. Espinosa et al. studied the I/D polymorphism of the ACE gene and the risk of ischemic heart disease. They found that the DD genotype is associated with MI in young patients, although smoking status showed a much more powerful association [14]. Butler et al. investigated the effects of cigarette smoking on each of the ACE genotypes [15]. They demonstrated that for both ID and DD genotypes, acetylcholine responses are blunted if the subjects were smokers. These data suggested that the DD ACE genotype in a young population is associated with a blunting of stimulated endothelial NO and donated NO responses [15]. The findings of the presented paper tend to support this hypothesis.

Cigarette smoking, increased fibrinogen levels and platelet activation, results in platelets and endothelium dysfunction that is implicated in vascular pathology. Membrane GP IIb/IIIa plays a major role in the platelet function, a key player in the development of thrombosis. In 1996, the presence of A2 allele was first reported to increase the risk of coronary heart disease [16]. Grove et al. investigate the relationship between the PLA1/A2 polymorphism and myocardial infarction in a large Scandinavian population [17]. The relationship between the number of PLA2 alleles and the risk of MI was found. They suggest significantly more frequent occurrence of A2 allele in patients with ischaemic heart disease than in healthy individuals [17]. They observed a weak interaction between smoking status and A2 allele. Lopes et al. noticed that smokers with the A2 allele were at greater risk for subsequent cardiac events (cardiac deaths, MI, refractory angina) in stable coronary artery disease [18]. According to Wiwanitkit, who performed metanalysis to assess the correlation between the pattern of GP IIb/IIIa polymorphism and MI, there is no association between this polymorphism and myocardial infarction [4].

In the presented study it was found that the percentage of patients with allele A2 were similar in non-smokers and smokers. Grove et al. found only a minor interaction between smoking and the PLA1/A2 polymorphism [17]. Ardissino et al. suggest a strong relationship between myocardial infarction and smokers bearing the A2 variant, compared to A2 negative non-smokers [19]. Grove et al. included in their analysis patients with both STEMI (ST-elevation myocardial infarction) and non-STEMI patients [17], while Ardissino et al. included young patients with STEMI only [19]. This may explain the different findings concerning smoking status and A2 variant of GPIIb/IIIa gene in both studies. The results of the presented study support data provided by Grove et al. suggesting that smoking could play a major and independent role in the pathogenesis of MI, with weak, if any, interaction with the GP IIb/IIIa gene PLA1/A2 polymorphism [17]. Further studies are needed to prove this hypothesis.

Endothelial dysfunction plays a pivotal role in the pathogenesis of myocardial infarction. The association of eNOS gene polymorphism (G894T) with MI has been confirmed by some studies [7, 8]. Shimasaki et al. examined the possible association of this eNOS gene polymorphism and myocardial infarction [7]. They observed a significant correlation between the missense T variant of eNOS gene and myocardial infarction. They suggest that the association may be due to the impaired effects of NO on the cardiovascular system: dysregulation of vascular tone, platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation. Leeson et al. investigated whether this polymorphism was associated with functional changes in the endothelium, and how the particular genotype alters the harmful or beneficial impacts of environmental influences on the endothelium [20]. They found that vascular function was not related to

a specific genotype in the whole study group, and within both genders. However, among males carrying T allele, smoking was associated with lower endothelium-dependent, flow-mediated, brachial artery dilatation [20]. Chang et al. investigated if this polymorphism in the eNOS gene locus is a definite risk factor for coronary spasm, and whether diffuse spasm of normal-looking coronary artery correlates significantly with G894T polymorphism, in contrast with a focal spasm superimposed on an atherosclerotic plaque [21]. A significantly higher incidence of the T polymorphic variant in patients affecting coronary spasm than in the control group. This result supports the hypothesis that diffuse coronary spasm is significantly associated with endothelial dysfunction in contrast to focal spasm [21]. The data obtained from the presented study seem to be in accordance with other results reported above.

Nowadays, gene-environment interactions in the pathogenesis of cardiovascular diseases are under extensive investigation. However, the multifactorial nature of these diseases makes this task extremely difficult and laborious.

CONCLUSIONS

1. Both angiotensin converting enzyme gene I/D and endothelial nitric oxide synthase gene G894T polymorphisms associations with myocardial infarction seem to be influenced by smoking habit.
2. The obtained results do not support such an impact of the platelet glycoprotein IIb/IIIa gene PLA1/A2 polymorphism.

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