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Original Research Article

Relationship of the rs1799752 polymorphism of the angiotensin-converting enzyme gene and the rs699 polymorphism of the angiotensinogen gene to the process of in-stent restenosis in a population of Polish patients with stable coronary artery disease

Tadeusz Osadnik [a,c,*], Joanna Katarzyna Strzelczyk [d], Martyna Fronczek [b,c], Kamil Bujak [b], Rafał Reguła [b], Małgorzata Gonera [b], Marcin Gawlita [b], Anna Kurek [b], Jarosław Wasilewski [b], Andrzej Lekston [b], Marek Gierlotka [b], Michał Hawranek [b], Zofia Ostrowska [d], Andrzej Wiczkowski [d], Lech Poloński [b], Mariusz Gąsior [b]

[a] 2nd Department of Cardiology and Angiology, Silesian Center for Heart Diseases, Zabrze, Poland
[b] 3rd Department of Cardiology, School of Medicine with the Division of Dentistry, Medical University of Silesia, Zabrze, Poland
[c] Genomics Laboratory, Kardio-Med Silesia Science and Technology Park, Zabrze, Poland
[d] Department of Medical and Molecular Biology, School of Medicine with the Division of Dentistry, Medical University of Silesia, Zabrze, Poland

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ABSTRACT

Purpose: The renin–angiotensin–aldosterone system may influence in-stent restenosis (ISR) via angiotensin II, which stimulates the production of growth factors for smooth muscle cells. The aim of this work is to assess the influence of the rs1799752 polymorphism of the angiotensin-converting enzyme (ACE) gene and the rs699 polymorphism of the angiotensinogen (AGT) gene on the ISR in Polish patients with stable coronary artery disease (SCAD) who underwent stent implantation.
Material/methods: Two hundred and sixty-five patients with SCAD were included in the study. All patients underwent stent implantation upon admission to the hospital and had subsequent coronary angiography performed. The patients were divided into two groups – those with significant ISR ($n = 53$) and those without ISR ($n = 212$). The ACE polymorphism was assessed using the classical PCR method and the AGT polymorphism was determined using the TaqMan method for SNP genotyping.
Results: No difference in the frequency of angiographically significant ISR occurrence associated with the different ACE and AGT gene polymorphisms was observed. In a multivariable analysis, after correction for clinical variables, the relationship between the ACE and AGT genotypes within the scope of the analyzed polymorphisms and the process of restenosis was not found using a dominant, recessive and log-additive model. Late lumen loss was also independent of the genotypes of the polymorphisms before and after correction with angiographic variables.
Conclusions: The rs1799752 polymorphism and the rs699 polymorphism had no relationship with the occurrence of angiographically significant ISR and late lumen loss in a group of Polish patients who underwent metal stent implantation.

1. Introduction

In-stent restenosis (ISR) is a re-narrowing of the vessel lumen by neointima growth after percutaneous coronary intervention. Based on histological examinations in the weeks after implantation of a metal stent, vascular smooth muscle cells comprise over 90% of neointima cells [1]. The renin–angiotensin–aldosterone system (RAAS) may influence ISR via angiotensin II, which stimulates the expression of platelet derived growth factor and fibroblast growth factor, potent growth factors for smooth muscle cells [2]. The key role in the conversion of angiotensin I to angiotensin II is played by angiotensin-converting enzyme (ACE) [3].

ACE concentrations in a given patient in time intervals are relatively constant; however, they show marked individual

* Corresponding author at: Silesian Centre for Heart Diseases, Marii Skłodowskiej-Curie 9, 41-800 Zabrze, Poland. Tel.: +48 32 373 36 19; fax: +48 32 273 26 79.
E-mail address: tadeusz.osadnik@sccs.pl (T. Osadnik).

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variability. Nearly 50% of ACE concentration variability may be explained in terms of the rs1799752 polymorphism; i.e., a deletion or insertion polymorphism (D/I polymorphism) of 287 base pairs in intron 16 of the ACE gene [4]. Angiotensin I, derived from the angiotensinogen (AGT) produced in the liver, is a substrate for ACE. The missense polymorphism rs699, which results in the substitution of methionine to threonine in a mature AGT molecule, has a moderate influence on its concentration – the AGT concentration in carriers of the C allele is higher by 10–20% [5].

The aim of this work was to assess the influence of the rs1799752 polymorphism of the ACE gene and the rs699 polymorphism of the AGT gene on the process of restenosis in Polish patients with stable coronary artery disease (SCAD) who underwent stent implantation. We have tested the best-known polymorphisms in AGT and ACE, to avoid too many intergroup comparisons. We have chosen rs1799752 based on its functionality as it was associated with higher ACE plasma level [4]. Rs699 has also been linked to predisposition to hypertension, coronary artery disease as well as atrial fibrillation [6–8].

2. Patients and methods

2.1. Patient population

The methods were described previously [9]. Briefly, we enrolled 265 SCAD patients with 322 lesions who underwent the implantation of at least one bare metal stent (BMS) and had subsequent coronary angiography performed because of a recurrence of angina or as a result of cardiac stress tests. The SCAD patients were included between 2007 and 2011. To our best knowledge patients were not related with each other. We used quantitative coronary angiography (QCA) for the exact assessment of coronary lesions, as well as minimal lumen diameter after BMS implantation and the stented segment after the next coronary angiography. QCA was assessed by 5 co-authors of the manuscript who had been randomly given angiograms. All angiograms were assessed by two cardiologists, measurement they agreed upon was entered into the database. All of them were blinded to genetic test result. Significant angiographic ISR was defined as a narrowing of the arterial lumen by >50% within the stented segment or up to 5 mm of the previously implanted BMS. We also assed the late lumen loss (LLL), which is an angiographic estimate of the extent of neointima formation. LLL was calculated as the difference between the minimal lumen diameter immediately after the procedure and the minimal lumen diameter after the next coronary angiography.

2.2. Genotyping

Total genomic DNA was extracted from 200 μl of whole blood samples using a GeneMatrix Quick Blood DNA Purification Kit (EUR_X, Poland). Genotyping of the ACE gene was assayed by polymerase chain reaction (PCR) using twenty-five nanograms of isolated genomic DNA; 0.2 μl 25 mM dNTP Mix; 1.2 μl 10 μM forward and reverse primers (Genomed, Poland); 2.5 μl DyNAzyme Buffer (ThermoScientific, USA); 0.3 μl DyNAzyme II DNA Polymerase (2 U/μl) (ThermoScientific, USA); and 17.6 μl DNase-, RNase-, and protease-free water (Qiagen, Germany).

The following primers were used: 5′CTGGAGACCACTCCCATCCT TTCT3′ in the forward direction and 5′GATGTGGCCATCACATT CGTCAGAT3′ in the reverse direction, as reported by Rigat et al. [10]. The DNA was amplified by initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 65 °C for 45 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min (Mastercycler personal, Eppendorf, Germany). The PCR products of the ACE gene were visualized on a 2% agarose gel with ethidium bromide staining. The ACE gene was classified as II, ID, or DD. The products were 490 bp (homozygous II) or 190 bp (homozygous DD). Heterozygous ID was confirmed by the presence of the two bands, 190 bp and 490 bp.

Due to the preferential amplification of the D allele, we repeated the PCR amplification under a different condition and using a primer pair which recognizes the insertion-specific sequence (in the forward direction: 5′TGGGACCACAGCGCCCGCCACTAC3′ and in the reverse direction: 5′TCGCCAGCCCTCCCATGCCCATAA3′), as reported by others [11].

The positive result of a 335 bp fragment was preset only for the I allele, thus confirming the ID genotype. The absence of the band was evidence of the DD genotype.

To minimize the risk of contamination, all three steps (isolation, amplification and electrophoresis) were conducted in three separate rooms. All samples were replicated twice.

The allele frequency was confirmed according to the Hardy–Weinberg equilibrium.

To identify single nucleotide polymorphisms of the AGT gene, we used a 5′ nuclease assay to discriminate between 2 alleles of a specific SNP. The identification of the AGT polymorphism was based on the transition/substitution of A/G alleles (rs699) using the context sequence of VIC and FAM: CAGGGTGCTGTCCA-CACTGGCTCCC[A/G]TCAGGGAGCAGCCAGTCTTCCATCC and Custom TaqMan SNP Genotyping Assays (Applied Biosystems, USA) according to the manufacturer's instructions with use of a TaqMan Genotyping Master Mix (No AmpErase UNG) (Applied Biosystems, USA) and 5 ng/μl of purified DNA. The reaction was conducted using the 7300 Real-Time PCR System (Applied Biosystems, USA). The samples were analyzed using SDS 1.4 software (Applied Biosystems, USA). For quality control, 10% of samples were randomly repeated, with complete congruence.

2.3. Statistical analysis

The comparison of continuous variables was performed using *U*-Mann–Whitney test with the exception of body mass index (BMI) variable, which was normally distributed and the intergroup comparison was performed using Student's *T*-test. The categorical variables were assessed using Fisher exact test. The Hardy–Weinberg equilibrium (HWE) for the genotypes was tested with the Chi-square test. The association between angiographically significant restenosis and the polymorphisms in the ACE and AGT genes in this group of patients ($n = 265$) was determined using dominant, recessive and log additive models that were corrected for clinical variables that reached a p-value of <0.3 in the group comparison [12]. The association of the LLL with the polymorphisms was calculated for lesions ($n = 322$) and corrected for angiographic variables that reached a p-value of <0.3 in the group comparison. The methodology to assess the patients and lesions separately is in concordance with the methodology used by other authors [13,14]. A value of $p < 0.05$ was considered to be statistically significant. Analyses were completed using the NCSS (Number Crunching Statistical Systems, Kaysville, USA) software and in the R environment using the SNPassoc package [15].

The study conformed to the declaration of Helsinki and was approved by the Ethics Committee of the Silesian Medical Chamber, Katowice, Poland (Approval No. 34/2011).

3. Results

3.1. Clinical and angiographic characteristics

The patients with ISR did not differ from patients without ISR in terms of age, sex, and co-morbidities, except for a more frequent occurrence of a myocardial infarction in the history in the group of patients with angiographically significant restenosis