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ASSOCIATION OF NOS3 GENE POLYMORPHISM rs1799983 WITH IN-STENT RESTENOSIS OF CORONARY ARTERIES

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Abstract

Revascularization by percutaneous coronary intervention (PCI) with stent implantation is an invasive technique commonly used in the therapy of acute coronary syndromes, including the myocardial infarction and other types of ischemic coronary disease. Although it is highly effective, some patients develop an in-stent restenosis requiring repeat percutaneous revascularization. Recent studies have indicated genetic association of single nucleotide polymorphisms in certain genes with the risk of restenosis.

The aim of this study was to investigate the association of NOS3 gene rs1799983 (Glu298Asp) polymorphism with the in-stent restenosis of implanted coronary stents.

In this prospective, observational, genetic-associated, case-control study demographic, clinical and laboratory data were analyzed from preliminary selected group of 77 patients with implanted coronary stents, of which 46 were with in-stent restenosis and 31 without restenosis. The gender and age distributions were similar among the two patient groups.

Using different genetic models, genetic association of NOS3 gene rs1799983 polymorphism and in-stent restenosis was revealed ($p<0.05$). Restenosis odds ratio indicated the protective role of the dominant G allele, while the minor variant T allele was associated with an increased risk of in-stent restenosis.

Keywords: in-stent restenosis, coronary stent, polymorphism gene, NOS3.

Introduction

Cardiovascular diseases, especially coronary artery disease (CAD), are a very important medical, public health, social and scientific problem with the highest morbidity and mortality in the developed world. Shortening of patients' life span, reduced quality of life, absence from work and treatment costs are additional consequences that have enormous social impact. Due to the high mortality and common complications, treatment of acute myocardial infarction has been a huge challenge for the medical practice as well as for the extensive scientific research.

Revascularization or reperfusion, which is made by percutaneous transluminal coronary angioplasty (PTCA), is a non-invasive, non-surgical, interventional technique that is most commonly used in the therapy of acute coronary syndromes including acute myocardial infarction as well as of stable angina and other forms of ischemic heart disease [1].

From a clinical point of view, it is necessary to distinguish between revascularization performed in cases of an incidental finding of stenosis during elective coronary angiography and revascularization indicated for acute myocardial infarction or in cases of a significant ischemia found during relevant examinations such as stress-test.

Restenosis is defined as a reduction in the diameter or narrowing of the lumen of the stent after its implantation in the coronary vessel by PTCA intervention.

Restenosis of the lumen of implanted stent has been one of the major complications in the interventional cardiology since its introduction of this modern treatment in clinical practice. In order to make a clinical definition of stent restenosis, it is necessary to have angiographic evidence of >50% stenosis of the artery diameter, but also presence of at least one of the following clinical symptoms: recurrent angina, objective signs of ischemia (such as relevant electrocardiographic changes), positive coronary hemodynamic assessment of the heart parameter FFR (fractional flow reserve) <0.08, minimum luminal surface of the coronary artery vessel evaluated with intravascular ultrasound (IVUS)

<4 mm^2 (and <6 mm^2 for left main artery) or restenosis with reduction of ≥70% of arterial lumen even in absence of clinical symptoms and signs [2].

Restenosis of the luminal coronary vessel after PTCA intervention can occur even without stent implantation, that is, after balloon dilatation, as well as after implantation of some of the available types of stents (BMS and DES) [3]. In the first case, lumen narrowing or complete occlusion can appear due to acute thrombosis of the blood vessel after balloon dilation or acute negative remodeling of the vessel wall or due to the phenomenon called elastic recoiling [3,4].

These mechanisms of restenosis occur early, during the intervention itself, and several hours or days later.

On the other hand, restenosis after stent implantation in the coronary vessel during PTCA intervention most commonly develops gradually over a longer period of several weeks or months and mainly because of excessive vessel intima proliferation. Therefore, this mechanism is called neointimal proliferation and is the most common reason for clinically relevant in-stent restenosis of implanted coronary stents [3,5].

Although the existence of certain clinical conditions such as diabetes are clearly associated with a higher incidence of restenosis development, it is obvious that it varies even in population of patients with relatively similar demographic and clinical characteristics. This speaks in favor of genetic association and is a subject of extensive clinical-genetic research.

The most common variants of the human genome are single nucleotide polymorphisms (SNPs). They occur, on average, in every 300 base pairs of the genomic DNA, which means there are about 10 million SNPs in the human genome. Their association with the risk of developing clinically relevant conditions and diseases is a subject of a vast number of conducted genetic-associated studies. In line with the general trends and technological development of human molecular genetics, research that examines the association of gene polymorphisms with restenosis has also evolved over the past two decades. The discovery of gene polymorphisms with confirmed clinical significance and sensitivity in prediction of the risk of restenosis has a potentially huge significance and high-priority research.

The NOS3 gene encodes the endothelial variant (type 3) of the enzyme nitric oxide synthase 3 (eNOS3), which contains 26 exons and is located in the region of chromosome 7 in humans (7q35-q36). Nitric oxide (NO) is a vasodilator molecule, which is known to inhibit thrombocyte adhesion and leukocyte extravasation through the blood vessel wall as well as to reduce proliferation and migration of vascular smooth muscle cells [6].

Among other polymorphisms, of clinical importance is the polymorphism rs1799983 (Glu298Asp). It is formed by a transversion of nucleotide G to T in the region of codon 894 in exon 7, resulting in the replacement of glutamic acid with asparagine acid in the protein product. This variant causes reduced enzyme activity and reduced NO production from the endothelial cells.

Pathophysiological role of the reduced nitric oxide levels in the stimulation of vascular smooth muscle cells proliferation has been known and has been confirmed in a large number of experimental studies [7].

Thus, polymorphisms that influence on the expression of nitric oxide synthase were a topic of investigation on the possible association with clinical restenosis twenty years ago.

Aims

The primary aim of this paper was to determine the association of nitric oxide synthase (NOS3) gene rs1799983 (Glu298Asp) polymorphism from one, and the frequency of restenosis after implantation by percutaneous transluminal coronary angioplasty due to coronary artery disease, from the other side.

Material and methods

So far, within the framework of this prospective, observational, genetic-associated study of cases and controls, genetic data of a total of 77 patients with implanted coronary stents have been analyzed. Of this number, 46 patients were with in-stent restenosis and 31 without restenosis. Patients were selected according to inclusion and exclusion criteria and had similar gender and age distribution.

Routine demographic, clinical and laboratory data and samples of up to 3 mL of venous blood with anticoagulant (disodium salt of ethylenediamine tetraacetic acid - EDTA.Na2) were collected from

patients who were treated at the University Clinic for Cardiology, with previously obtained written consent by the professional staff of the Clinic, along with a written informed consent from each patient.

The study was approved by the Ethics Committee of the Faculty of Medicine in Skopje. In addition, patients' information was analyzed with confidence and in line with the Law on personal data protection.

Isolation of the genomic DNA was made with the method of sodium chloride salting, chloroform extraction and subsequent ethanol precipitation. Molecular analyses for determination of the NOS3 gene rs1799983 (Glu298Asp) polymorphism were made by genotyping with TaqMan fluorescence probes with nucleotide sequence specific for the amplified region of the relevant gene, labelled at 5'-end with fluorescence dye FAM or VIC, while at 3'-end with NFQ quencher.

The curves obtained were read with the software StepOne (Applied Biosistems). Molecular-genetic analyses were done in the Laboratory for molecular biology at the Faculty of Natural and Mathematical Sciences in Skopje.

The frequencies of genotypes and of alleles of the gene polymorphism were analyzed with the Fischer's exact test by using the genotypic, allelic, dominant, recessive, heterozygous and super-dominant models. Allelic frequencies were analyzed with the allelic and additive models and Cochran–Armitage trend-test was used. At the same time odds ratio (OD) with confidence interval (CI) of 95% was determined. The value of $p<0.05$ was considered statistically significant and the value of $p<0.01$ highly significant.

Statistical analysis was done by using the software programs XLSTAT 2016, GenAlEx 6.5 and Microsoft Excel 2016.

Results

This paper presents only the preliminary results obtained from the statistical analysis of the relevant demographic, clinical and laboratory parameters of 77 patients.

Gender distribution in groups of patients with and without restenosis of implanted coronary stents are presented in Table 1.

Table 1. Gender distribution of patients.

Gender (n=77)	**Group with restenosis**		**Group without restenosis**		**Fisher's exact test ***
	n	**%**	**n**	**%**	***p***
male	35	76.09	23	74.19	1.000
female	11	23.91	8	25.81	
total	**46**	**100.00**	**31**	**100.00**	

* two-way

According to the presented data, about ¾ of patients from both groups were men. There was no statistically significant difference in gender distribution between two groups of patients ($p>0.05$).

Age distribution in both groups of patients is shown in Table 2 and Figure 1.

According to the presented results, there was a high similarity regarding the mean, minimum and maximum values as well as a standard deviation of the age value of patients from both groups, and the differences were statistically non-significant ($p>0.05$).

Table 2. Age distribution of patients.

Parameter (years)	Group with restenosis	Group without restenosis	Student's t-test *
n	46	31	0,914
mean	64,59	64,35	
SD	9,86	8,45	
min. age	47	47	
max. age	85	79	

* two-way; SD=standard deviation

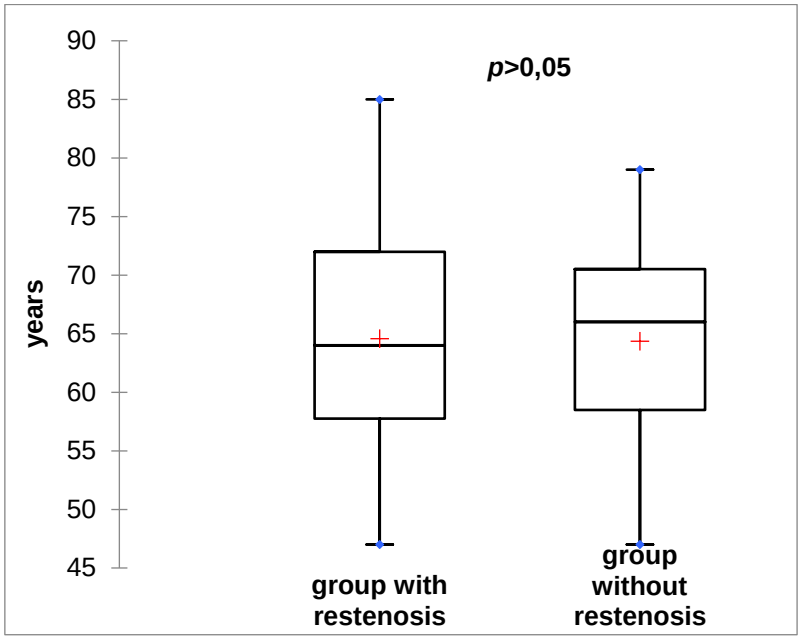

Figure 1. Difference in age distribution between groups of patients.

According to the presented results, there was a high similarity regarding the mean, minimum and maximum values as well as a standard deviation of the age values of patients from both groups, and the differences were non-significant ($p>0.05$).

The absence of significant differences between the group with and the group without restenosis regarding gender and age structure has shown that they were well balanced and that these demographic parameters could not have a negative impact on the results' interpretation from genetic analysis.

Furthermore, a series of statistical analyses for assessment of possible existence of genetic correlation of the examined NOS3 gene rs1799983 polymorphism with in-stent restenosis of coronary arteries were made.

The comparison of genotypic frequencies was made by using several genetic models, and the results are illustrated in Table 3.

When comparing the genotypic combinations, that is, frequencies of carriers of the dominant genotype GG versus the sum of frequencies of carriers of the heterozygous genotype GT and the variant genotype TT, a statistically significant difference was observed when using the dominant, allelic and additive models ($p<0.05$). The calculated odds ratio has shown that the carriers of the dominant genotype GG had a 0.387-fold smaller chance to develop restenosis than the carriers of the genotypes GT and TT. The reverse formulated calculation (1-0.450=0.613) has demonstrated that individuals with genotypes GT and TT had a 61.3% higher likelihood of restenosis than those with genotype GG. The frequency of the minor allele T was 34.78% in the group with restenosis versus 19.35% in the group without restenosis, which was statistically significant ($p<0.05$).

Additive model has indicated that the likelihood of restenosis increased with the number of T alleles in the genotype of the carriers. There were no statistically significant differences by applying the other genetic models for comparison of genotypic frequencies (p>0.05).

Table 3. Differences in the frequencies of genotypes and alleles of NOS3 gene rs1799983 polymorphism between groups with and without restenosis.

Genetic model	**NOS3 rs1799983 genotype/allele**	**Group with restenosis**		**Group without restenosis**		**Fisher's-exact test**	***p***	***OR* (95% CI)**
		n	**%**	**n**	**%**			
Genotypic	**GG**	19	41.30	20	64.52	4.439	0.138	Reference values
	GT	22	47.83	10	32.26			0.432 (0.163 - 1.146)
	TT	5	10.87	1	3.23			0.190 (0.020 - 1.779)
	Total	46	100.00	31	100.00			
Dominant	**GG**	19	41.30	20	64.52	3.992	**0.046**	0.387 (0.151 - 0.992)
	GT + TT	27	58.70	11	35.48			
	Total	46	100.00	31	100.00			
Recessive	**GG + GT**	41	89.13	30	96.77	1.506	0.220	0.273 (0.030 - 2.462)
	TT	5	10.87	1	3.23			
	Total	46	100.00	31	100.00			
Heterozygous	**GG**	19	46.34	20	66.67	2.891	0.089	0.432 (0.163 - 1.146)
	GT	22	53.66	10	33.33			
	Total	41	100.00	30	100.00			
Super-dominant	**GG + TT**	24	52.17	21	67.74	1.848	0.174	0.519 (0.201 - 1.343)
	GT	22	47.83	10	32.26			
	Total	46	100.00	31	100.00			
Allelic	**G**	60	65.22	50	80.65	4.320	**0.038**	0.450 (0.210 - 0.964)
	T	32	34.78	12	19.35			
	Total	92	100.00	62	100.00			
Additive	**0 T**	19	41.30	20	64.52	2.098 **	**0.036**	/
	1 T	22	47.83	10	32.26			
	2 T	5	10.87	1	3.23			
	Total	46	100.00	31	100.00			

***** two-way

****** two-way Cochran-Armitage ordinary test

Discussion

According to the results obtained so far in this study, NOS3 gene rs1799983 polymorphism is associated with the development of in-stent restenosis of coronary arteries. This genetic association was confirmed with many genetic models and was statistically significant ($p<0.05$). Odds ratio index for onset of restenosis has indicated the protective role of the dominant allele G versus the minor, variant allele T, which has been associated with the risk of stent restenosis.

Previous studies, such as that of Suzuki *et al.* (2002) and Gomma *et al.* (2002) revealed a clear genetic association of NOS3 gene rs1799983 polymorphism (Glu298Asp) with the increased risk of restenosis [8,9]. These authors, independently of each other, think that this genetic polymorphism is an independent predictor for in-stent restenosis.

Similar results have been obtained in other more recent studies. For example, the genetic study conducted by Shuvalova *et al*. (2012) analyzed several polymorphisms in cases with restenosis and in a control group of patients without restenosis [10]. The analysis revealed that the minor T-allele of NOS3 gene Glu298Asp polymorphism was significantly associated with an increased risk of in-stent restenosis.

In the clinical genetic-associated study of Zholdybayeva *et al.* (2016), a total of 459 patients with restenosis after coronary artery stenting were examined [11]. Among a total of 53 genotyped SNP polymorphisms, the most significant change in odds ratio for developing restenosis was found in NOS3 gene rs1799983 (Glu298Asp) polymorphism, for OR=20.05; p=2.74 x 10-12 calculated with additive model, OR=22.24; p=6.811 x 10-10 with recessive genetic model.

Contrary to this, the retrospective study of Zeng *et al*. (2017) included 425 patients who had previously undergone revascularization and implantation of DES stent [12]. Their analyses showed absence of significant association between in-stent restenosis and NOS3 gene rs1799983 (Glu298Asp) polymorphism. However, it has to be emphasized that the authors found a correlation with another polymorphism (T786C) in the same gene.

Further research will help in better clarifying the role of this and other two polymorphisms in risk of developing in-stent restenosis.

Conclusion

Clinical usage of such genetic markers can play a key role in the individual approach to each and every patient when making a selection of the stent and bringing other periprocedural decisions as well as in selection of post-PTCA therapy, prognosis and follow-up of the disease in stented patients.

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