Original Research Article

Association of Glu298Asp polymorphism of eNOS gene with cardiovascular diseases

ABSTRACT

Molecular-genetic diagnostics of polygenic diseases is a new and interesting area in laboratory diagnostics, especially in the area of cardiovascular diseases, as one of the leading causes of mortality in the world population.

Aims: The aim of the paper was to analyse variants of the endothelial nitric oxide synthase gene (NOS3) (Glu298Asp/G894T) in the human population of Tuzla Canton in relation to cardiovascular diseases.

Study design: The study included 112 respondents of both sexes over 18 years old. The experimental group for the analysis of the polymorphism (Glu298Asp) of the endothelial nitric oxide synthase gene included 56 respondents of both sexes with cardiovascular disease (hypertension), while the control group comprised 56 healthy respondents of both sexes without a prior history of cardiovascular disease (sample/control).

Place and Duration of Study: Blood sampling was performed at Medical Center "Plava Poliklinika", "Plava Medical Group", Department of Biochemistry, Microbiology and Genetics, Tuzla. DNA isolation and molecular-genetic analysis of the samples were performed in Laboratory for scientific research at the Department of Biology, Faculty of Natural Sciences and Mathematics in Tuzla.

Methodology: The genotyping of eNOS Glu298Asp polymorphism for all respondents was determined by an optimized method based on PCR-RFLP reaction.

Results: In the total sample of respondents, the highest genotype frequencies of the eNOS gene were recorded for the GG genotype (53.5%) and the GT genotype (35.7%). The lowest frequency was recorded for the TT genotype, which was 10.8%.

Conclusion: The results obtained in the study provide good guidelines for further study of a molecular-genetic association between a high number of gene candidates and cardiovascular diseases, which will contribute to the incorporation of these results into the existing regional and European genetic database.

Keywords: polymorphism, NOS3, cardiovascular diseases, Tuzla Canton

1. INTRODUCTION

Endothelial nitric oxide synthase (NOS3 or eNOS) is the enzyme responsible for the highest production of nitric oxide, with the great impact on the cardiovascular system, encoded by the eNOS gene, which presents various polymorphisms [1]. Understanding the nitric oxide molecular mechanisms of signaling pathways in the heart can provide a new strategic approach to prevention and treatment of diseases related to the cardiovascular system [2].

Shankarishan et al., (2014) citied that have been described a large number of polymorphisms of the eNOS gene, but that the polymorphisms of intron 4 a/b, exon 7 Glu298Asp (rs 1799983) and T786C (rs 2070744) the eNOS gene are the most studied in relation to the association with hypertension [3].

Abdel-Aziz et al., (2013) in the study of the association of polymorphisms of the eNOS gene with the risk of early coronary artery disease concluded that the investigated polymorphisms are risky. Hypertension, diabetes, smoking, total cholesterol, triglycerides, LDLc, HDLc and TT genotype of the Glu298Asp polymorphism of the eNOS gene were independent risk factors for the development of premature coronary disease [4].

Ogretmen et al. in their preliminary study on the association of eNOS Glu298Asp gene polymorphism in psoriasis cases with hypertension in the Turkish population analyzed the genetic profiles of 75 patients with psoriasis (21 hypertensive and 54 normotensive) and 55 healthy (normotensive and non-psoriatic) subjects. The group with psoriasis had a frequency of 50.7% (38) for the GG genotype, 42.7% (32) for the GT genotype and 6.6% (5) for the TT genotype. In the control group, the frequencies of the GG, GT and TT genotypes were (39) 70.9%, (15) 27.2% and (1) 1.8%, respectively. An increased relative T allele frequency in eNOS Glu298Asp polymorphism of (42) 28.0% was determined in psoriatic patients when compared with normotensive healthy respondents without psoriasis with a frequency of (27) 15.4%. The G allele frequency was (108) 72.0% in the group with psoriasis, while in the control group it was (93) 84.5% [OR=2.1, 95%CI (1.14–3.99); (p=.017)]. Their preliminary results indicated that there was a correlation between eNOS Glu298Asp polymorphism and hypertension among psoriatic patients in the Turkish population. The authors also suggest that these results need to be confirmed by more extensive studies [5].

In the population of Tuzla Canton, to date has not been performed genetic studies and/ormolecular genetic characterization of eNOS Glu298Asp polymorphism. Thus, it was important to conduct molecular-genetic testing to provide evaluation of the genotype distribution of the eNOS gene in respondents with cardiovascular diseases (CVD) as well as in the control group.

Aims

The primary aim of the study was the determination of allele and genotype frequencies of the endothelial nitric oxide synthase (NOS3) in the human population of Tuzla Canton and the accurate assessment of the association of the Glu298Asp/G894T polymorphic variant of the endothelial nitric oxide synthase gene with cardiovascular diseases.

2. MATERIAL AND METHODS

2.1. Respondents

The study included 112 respondents of both sexes over 18 years old. The experimental group for the analysis of the polymorphism (Glu298Asp) of the endothelial nitric oxide synthase gene included 56 respondents of both sexes with cardiovascular disease (hypertension), while the control group comprised 56 healthy respondents of both sexes without a prior history of cardiovascular disease (sample/control).

Blood sampling was performed at Medical Center "Plava Poliklinika", “Plava Medical Group”, Department of Biochemistry, Microbiology and Genetics, Tuzla. The study was conducted in accordance with the ethical principles as per the Declaration of Helsinki.

All participants were informed on the study protocol and gave their signed consent to participate in this study by supplying a sample for DNA isolation and molecular genetic characterization of eNOS gene genotypes.

2.1.1. Samples

For DNA isolation and analysis, 3 mL of peripheral blood was collected in tubes with EDTA anticoagulant (Vacutainer Becton Dickinson, Meylan Cedex, France and BD Vacutainer K2E, BD-Plymouth, PL6 7 BP. UK). Respondents selection and blood sampling for the study were conducted at Medical Center "Plava Poliklinika", "Plava Medical Group", Department of Biochemistry, Microbiology and Genetics, Tuzla with the approval of the Ethics Committee of the healthcare institution (Number: 1627-3/21).

2.1.2. Molecular-genetic analysis

DNA isolation and molecular-genetic analysis of the samples were performed in Laboratory for scientific research at the Department of Biology, Faculty of Natural Sciences and Mathematics in Tuzla. For DNA isolation and analysis, the respondents' peripheral blood samples were used, collected from the tubes with EDTA anticoagulant (Vacutainer Becton Dickinson, Meylan Cedex, France). DNA isolation was performed using a commercial kit (Qiagen GmbH. Hilden, Germany) according to the manufacturer's instructions.

The genotyping of eNOS Glu298Asp polymorphism for all respondents was determined by an optimized method based on PCR-RFLP, using primers 5'-CATGAGGCTCAGCCCCAGAAC-3' (sense) and 5'-AGTCAATCCCTTTGGTGCTCAC-3' (antisense) [6]. The amplification was done using a polymerase chain reaction machine (Applied biosystems by life technologies, 2720 Thermal Cycler). The PCR mixture in 25 µL of final volume contained 10.5 µL of deH_2O (Sigma Aldrich), 0.5 µL of each primer, 12.5 µL of REDTaq® ReadyMix™ and 100 ng of genome DNA (1 µL of DNA). Initial denaturation was conducted at 95ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing primers at 60ºC for 30 s and elongation at 72º C for 30 s [4, 7].

Restriction digestion was performed with the enzyme MboI (New England Biolab) [4, 6, 7]. The PCR and restriction digestion products were separated by electrophoresis on 3% agarose gel. Visualization of the obtained fragments was detected by a UV transilluminator (VWR GenoMini, VWR International, BVBA Lueven). The difference between genotypes was determined by the analysis of the obtained restriction fragments with the length of 87, 119 and 206 bp, using relevant DNA markers of known size.

2.1.3. Statistical Data Analysis

The χ^2 test with one degree of freedom was used for the analysis of the distribution of the absolute and relative genotype frequencies and deviation from Hardy-Weinerberg equilibrium (HWE). The results were considered statistically significant when the p-value was <.05. The degree of association among the studied groups was evaluated using odds ratios with a confidence interval of 95% [OR(95% CI)].

3. RESULTS AND DISCUSSION

The study included a total of 112 individuals divided into two groups – a group with CVD (hypertension) and a control group. In both groups were 56 individuals of both sexes from Tuzla Canton. Allele and genotype frequencies of eNOS Glu298Asp polymorphism were determined. Furthermore, the accurate assessment of the association of the

Glu298Asp/G894T polymorphic variant of the endothelial nitric oxide synthase gene with cardiovascular disease was conducted in the studied population. Table 1 shows the absolute and relative genotype frequencies of the eNOS gene (Glu298Asp) in the total sample and subsamples.

In the group with CVD, the highest frequency was recorded for the GG genotype and it was 50.0%. The GT genotype was determined in 34.0% of the participants and the TT genotype in 16.0% of the individuals of this group. In the control group, most participants had the GG genotype (57.1%). The GT genotype was present in 37.5% of the individuals and 5.4% of the participants had the TT genotype (Table 1).

The analysis of statistical significance of genotype frequencies between the groups did not indicate any significant differences (p>.05). The results are shown in Table 1. The allele frequencies and their distribution in the total sample and subsamples were analyzed. In the group of CVD in the sample of 56 participants, the G allele frequency was 67.0% and the T allele frequency was 33.0%. In the control group in the sample of 56 individuals, the G allele frequency was 76.0% and the T allele frequency was 24.0%. In the total sample of 112 participants, the G allele frequency was 71.4% and the T allele frequency was 28.0% (Table 2).

Table 1. Absolute and relative genotype frequencies of eNOS gene (Glu298Asp) in total sample and subsamples

		CVD		Total	χ^2	df	p
Genotype	**N(%)**	**Yes**	**No**				
GG	N (%)	28 (50.0%)	32 (57.1%)	60 (53.5%)			
GT	N (%)	19 (34.0)	21 (37.5%)	40 (35.7%)			
TT	N (%)	9 (16.0)%)	3 (5.4%)	12 (10.8%)	3.367	2	.186
Total	N	56	56	112			
Genotype	N	**CVD**					
		Yes	**No**	Total	χ^2*	**df***	**p***
GG	N (%)	28 (50.0%)	32 (57.1%)	60 (53.5%)			
GT+TT	N (%)	28 (50.0%)	24 (42.9)	52 (46.5%)	.574	1	.448
Total	N	56	56	112			
					OR(95% CI)*	**z stastistic***	**p***
					1.3333 (.6332-2.8076)	.757	.4489

*CVD= cardiovascular disease

*For GG genotype in relation to GT+TT

Table 2 shows the analysis of the allele frequencies and their distribution in the total sample and subsamples.

Table 2. Absolute and relative allele frequencies of eNOS gene (Glu298Asp) in total sample and subsamples

	CVD			χ^2	df	p
	Yes	**No**	**Total**			

Allele (G)	75 (67.0%)	85 (76.0%)	160 (71.4%)			
Allele (T)	37 (33.0%)	27 (24.0%)	64 (28.6%)	2.1875	1	.139
Total	112	112	224	**OR(95% CI)***	**z stastistic***	**p***
				1.5531 (.8650-2.7885)	1.474	.1404

The genotype and allele frequencies of the eNOS gene were analyzed according to sex.

Table 3. Absolute and relative genotype frequencies of eNOS gene (Glu298Asp) in total sample and subsamples according to sex

Genotype		**Sex**		χ^2	**df**	**p**
		Female	**Male**			
GG	**N (%)**	32 (53.3%)	28 (53.85%)			
GT	**N (%)**	25 (41.7%)	15 (28.85%)			
TT	**N (%)**	3 (5.0%)	9 (17.3%)	5.222	2	.073
Total	**N**	60	52			
Genotype	**N**	Female	Male	χ^2*	**df***	**p***
GG	**N (%)**	32 (53.3%)	28 (53.85%)			
GT+TT	**N (%)**	28 (46.7%)	24 (46.15%)	.0029	1	.956
Total	**N**	60	52			
				OR(95% CI)*	**z stastistic***	**p***
				1.0208 (.4848-2.1496)	.054	.9567

***For GG genotype in relation to GT+TT

The study included 60 female respondents and 52 male respondents (Tables 3 and 4).

Table 4. Absolute and relative allele frequencies of eNOS gene (Glu298Asp) in subsamples according to sex

	Sex			χ^2	**df**	**p**
Alleles	**Female N=60**	**Male N=52**	**Total N=112**			
Allele (G)	89 (74.2%)	71 (68.3%)	160 (71.4%)			
Allele (T)	31 (25.8%)	33 (31.7%)	64 (28.6%)	.949	1	.330
Total	120	104	224			
				OR(95% CI)*	**z stastistic***	**p***
				0.7494 (.4192-1.3399)	.973	.3305

The genotype distribution of the eNOS gene (Glu298Asp) was analyzed according to the ≤44 i ≥45 age classes for the GG genotype in relation to GT+TT (Table 5).

Table 5. Genotype distribution of eNOS gene (Glu298Asp) according to age classes ≤44 and ≥45

Genotype		Age classes			χ^2	df	p
		≤44	≥45	Total			
GG	N (%)	38 (56.72%)	22 (48.89%)	60 (53.57%)			
GT+TT	N (%)	29 (43.28 %)	23 (51.11%)	52 (46.43%)	.663	1	.415
Total	N	67	45	112			
					OR(95% CI)	z stastistic	p
					.7300 (.3419-1.5584)	.813	.4160

The comparison of the eNOS gene genotype (Glu298Asp) according to the ≤44 and ≥45 age classes for the GG genotype in relation to GT+TT did not indicate any significant differences in the distribution (Table 5).

In the study in the total sample of respondents, a higher frequency of the TT genotype (16.0%) was determined in the group with CVD compared with the control group where the recorded frequency was 5.4%. However, the differences in the genotype distribution of the eNOS gene polymorphism (Glu298Asp) were not significant.

Nassereddine et al. in the study on the association of G894T eNOS polymorphism with susceptibility to essential hypertension in Morocco determined the GG genotype in (5) 3.49% of the respondents in the group with hypertension and in (116) 63.04% of the respondents in the control group. For the GT genotype, the frequency was (54) 37.24% in the group of respondents with hypertension and (62) 33.69% in the control group. The TT genotype was determined in (86) 59.27% of the respondents with hypertension and (6) 3.27% of the respondents in the control group. A statistically significant difference was determined by comparing GT genotype frequencies in the group of respondents with hypertension and in the control group [OR=20.2; 95%CI (7.7-52.4); (p= <.0001)]. Furthermore, a statistically significant difference was determined by comparing TT genotype frequencies in the group of respondents with hypertension and in the control group [OR=332.5; 95%CI (98.2-1125.4); (p=<.0001)]. This study indicated a strong association of G894T eNOS polymorphism with essential hypertension in the Moroccan population [8].

Jiménez-González et al. in the study on identification of genetic risk factors associated with ischemic stroke in the Mexican population observed the Glu/Glu, Glu/Asp and Asp/Asp frequencies of (107) 52.4%, (86) 42.2% and (11) 5.4% respectively in the group with hypertension. In the control group, the Glu/Glu, Glu/Asp and Asp/Asp frequencies were (146) 71.6%, (52) 25.5%, and (6) 2.9% respectively. In the group with CVD, the Glu/Glu frequencies were (107) 52.4% in comparison to the Glu/Asp+Asp/Asp frequencies which were (97) 47.6%. In the control group, the Glu/Glu frequencies were (146) 71.6% in comparison to the Glu/Asp+Asp/Asp frequencies which were (58) 28.4%. Significant differences were determined in distribution by comparing the group with ischemic stroke and the control group (p=.001) [9].

Kumar et al. in a meta analysis of 27 studies (patients-controls) involving 6733 patients and 7305 controls, indicated a significant association of G894T [OR=1.17, 95% CI (1.08-1.28); (p<.001)] and 4b/a [OR=1.25, 95% CI (1.13-1.39); (p<.001)], while the association was not statistically significant for the T786C [OR=1.11, 95% CI (0.98-1.26); (p=.109)] polymorphism of the eNOS gene and ischemic heart diseases. This meta analysis indicates a significant association of the G894T and 4b/a polymorphisms of the eNOS gene with the risk of ischemic heart disease. However, there was no association of the T786C polymorphism of the eNOS gene with the risk of ischemic heart disease (differences were not statistically significant). The authors indicate that additional studies with larger sample size are required for the confirmation of these results [10].

Srivastava et al. in the study on the association of eNOS (Glu298Asp) gene polymorphism with essential hypertension in Asian Indians determined higher prevalence of the GT+TT genotypes in patients than in controls as well as the association of T allele with essential

hypertension [OR=2.10, 95%/CI (1.34-3.28); (p<.001)]. The study suggested the respondents in this area with T allele to be at higher risk to develop essential hypertension. The analysis of the genotype distribution according to sex in males with essential hypertension identified the GG genotype in (103) 61.3% of the respondents, the GT genotype in (61) 36.3% of the respondents, and the TT genotype in (4) 2.4% of the respondents. In the control group, the GG, GT and TT genotypes were determined in (124) 77.0%, (35) 21.8% and (2) 1.2% of the respondents, respectively [OR=2.11; 95%/CI= (1.27–3.52); (p<.01)]. In the group of female respondents with essential hypertension, the GG, GT and TT genotypes were determined in (35) 60.4%, (22) 37.9% and (1) 1.7% of the respondents, respectively. In female controls, the GG and GT genotypes were determined in (29) 74.4% and (10) 25.6% of the respondents, while the TT genotype was not recorded in this group (0) 0% of the respondents). The odds ratio showed the following results: [OR=1.91, 95%/CI= (0.72–5.12); (p=.31)] [11].

Djuric et al. in the study conducted in Serbia determined the GG, GT and TT genotypes in (99) 43.8%, (101) 44.7% and (26) 11.5% of the respondents, respectively, in the group of patients with carotid atherosclerosis. In the control group, the GG, GT and TT genotypes were determined in (135) 47.2%, (122) 42.7% and (29) 10.1% of the respondents. The analysis of statistical significance did not indicate any significant differences comparing patients and controls. For the GG, GT and TT genotypes in patient/control group, p values were 0.44, 0.61 and 0.53, respectively. They conclude that Glu298Asp eNOS gene polymorphism by itself is not associated with the occurrence of carotid plaque in Serbian patients with carotid atherosclerosis. However, further association studies with emphasis on the effect of haplotype need to be conducted [12].

Velloso et al., (2010) in their analysis of the eNOS gene polymorphism determined that the genotype frequency for Glu298Glu was (49) 49.0% in patients with CVD and (36) 35.0% in the control group (p=.030). The Glu298 Asp genotype had a frequency of (47) 47.0% in patients with CVD and (51) 49.5% in the control group (p=.414). The Asp298Asp genotype had a frequency of (4) 4.0% in patients and (16) 15.5% in the control group (p=.005) [13].

Đurić et al. in their study determined that Asp298Asp genotype could be a significant marker for interindividual differences in risk for complicated plaque development in Serbian patients with advanced atherosclerosis. However, they concluded that the number of patients included in this study was limited to make a final conclusion and that this result must be validated in a larger number of patients from Serbia as well as in other populations [14].

4. CONCLUSION

Multifactorial diseases such as cardiovascular ones are linked to genetic and environmental factors. Studies on the risk of their occurrence involves complex, multifactorial research on molecular-genetic level which could enable the assessment of risk profiles. Since it is not known if studies on the effect of the eNOS gene variations (the Glu298Asp variant) on the susceptibility to cardiovascular diseases have been conducted in our area, this study provides good guidelines for further study and development of molecular-genetic diagnostics and recommended genetic testing in the assessment of risk factors for the development of cardiovascular diseases in health care institutions in Tuzla Canton and more broadly across the region. The results of this study could be a basis for the application in molecular clinical diagnostics, the informativeness assessment of endothelial nitric oxide synthase gene polymorphism (Glu298Asp, G894T) as an adequate marker in molecular diagnostics. The realization of this study has offered preliminary results of the molecular genetic characterization of the eNOS gene in the human population of Tuzla Canton and the Bosnian-Herzegovinian population. The mentioned polymorphism may not be considered the main risk factor for hypertension. However, the results of this study indicate that its effect on hypertension needs to be investigated together with other genetic and acquired risk factors for cardiovascular diseases. Future studies will definitely cover other cardiovascular

diseases, a larger number of respondents and the association of anthropometric and biochemical parameters with genotype frequencies of endothelial nitric oxide synthase gene polymorphism (Glu298Asp).

CONSENT

All authors declare that all participants were informed on the study protocol and gave their signed consent to participate in this study by supplying a sample for DNA isolation and molecular genetic characterization of eNOS gene genotypes.

ETHICAL APPROVAL

The study was conducted in accordance with the ethical principles as per the Declaration of Helsinki. Respondents selection and blood sampling for the study were conducted at Medical Center "Plava Poliklinika", “Plava Medical Group”, Department of Biochemistry, Microbiology and Genetics, Tuzla with the approval of the Ethics Committee of the healthcare institution (Number:1627-3/21).

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