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MOLECULAR-GENETIC TYPING OF VNTR POLYMORPHISM eNOS GENE IN HUMAN POPULATION OF TUZLA CANTON

Original scientific paper

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ABSTRACT

The aim of the paper is to present the results of the VNTR gene polymorphism genetic variants molecular typing for endothelial nitric oxide synthase (eNOS) in human population of Tuzla Canton. Based on the analysis of the distribution of eNOS gene genotypes in the total sample of respondents, the highest frequency was recorded for the (b/b) genotype, which was 73.0%. For the heterozygous (a/b) genotype of the eNOS gene, a frequency of 24.0% was determined, and a frequency of 3.0% was recorded for the (a/a) genotype. The research resulted in a database of local and global significance, namely, the incorporation of these data into the existing regional and European genetic database.
Keywords: *Polymorphism, eNOS, Tuzla Canton*

INTRODUCTION

There is a great number of candidate genes that are associated with the development and occurrence of complex diseases. With regard to the significance of molecular research and diagnostics related to different types of diseases or predispositions for different diseases, the aim of this paper was the determination of allele and genotype frequencies of the eNOS gene in human population of Tuzla Canton. The presence of eNOS gene genotypes was analyzed according to sex and age groups of the respondents.

Nitric oxide (NO) is a vasoactive substance that is synthesised from L-arginine by neuronal (NOS1), endothelial (NOS3) and inducible (NOS2) nitric oxide synthases (Oliveira-Paula et al., 2016). Endothelial NOS (eNOS) is one of three isoforms of nitric oxide synthase enzymes (NOS), which is very important for maintaining the production of nitric oxide (NO), namely, for the regulation of cardiovascular homeostasis (Petranovic, 2013). Reduced production or activity of NO directly contributes to heart dysfunction (Thomas et al., 2013; Petranovic, 2013).

NOS3 gene is located in the 7q35-7q36 chromosome region of 7-th human chromosome (Marsden et al., 1993). There have been several polymorphisms of the eNOS gene of which three indicate the correlation with disease pathophysiology (Petranovic, 2013).

The role of eNOS gene three clinically significant polymorphisms: variable number of tandem repeats of 27 bp in intron 4 (VNTR 4b/a) (the longer allele has five tandem repeats of 27 bp (4b) while the shorter allele has four repeats (4a) (rs61722009)), a thymine (T) to cytosine (C) substitution at position 786 (rs2070744), and a guanine (G) to thymine (T) substitution in exon 7 at nucleotide position 894 (Glu298Asp) (rs1799983) has been the subject of various studies (Aziz Abbas et al., 2016; Thameem et al., 2008; Sivri et al., 2014; Afef et al., 2016).

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MATERIALS AND METHODS

The study included 100 male and female respondents aged above 18 from the local population of Tuzla Canton. The study was conducted in accordance with the ethical principles as per the Declaration of Helsinki of the World Health Organization (WHO). One part of the molecular analysis, the isolation of the deoxyribonucleic acid sample (DNA) was conducted in the Laboratory for Genetics and Microbiology at the department of Biology, Faculty of Natural Sciences and Mathematics, University of Tuzla, while the molecular genetic DNA typing was performed in the Laboratory for Human Genetics at the Institute for Genetic Engineering and Biotechnology (INGEB), University of Sarajevo. Total genomic DNA was isolated from EDTA anti-coagulated whole blood using the FlexiGene DNA isolation Kit (Qiagen, GmbH, Hilden, Germany). Using the polymerase chain reaction, DNA segments of the eNOS gene were amplified according to the protocol (Ramírez-Patiño et al., 2013) that have been additionally optimized. The in vitro synthesis reaction was performed on a device for the polymerase chain reaction (Applied Biosystems, GeneAmp PCR System 9700) and Mastercycler gradient (Eppendorf, Njemačka). The PCR reaction of the final volume of 10 µL contained 100 ng of the genome's DNA, 5 µL of REDTaq® ReadyMix™ PCR Reaction Mix (Sigma Aldrich, Munich, Germany), 0,2 µL of the primer (5′ AGG CCC TAT GGT AGT GCC TTT-3′ and 5′-TCT CTT AGT GCT GTG GTC AC-3′) (Ramirez-Patino et al., 2013) and 3,6 µL of sterile deionized water. The examination of the amplified PCR products of the eNOS gene was done by electrophoresis on a 2% agarose gel to which 5 µL of Midori Green were added (Nippon Genetics Europe GmbH, Germany). In order to separate the amplified products, an ABI PRISM ®3500 automatic sequencer (Genetic Analyser 3500, Applied Biosystems) was used, with eight capillaries 50 cm long, which enabled the separation and analysis of DNA fragments on the basis of capillary electrophoresis as well as detection by a CCD camera. ABI PRISM® PRISM Genotyper Software was used for the analysis and interpretation of the observed loci, which enabled the quantification and conversion of the results into the results for the genetic database.

Deviation of allele and genotype distribution from the Hardy-Weinberg equilibrium was assessed by the (χ2) chi square test. Statistical significance was set at a P value of $< .05$.

RESULTS

The study included 100 healthy male and female respondents from the local population of Tuzla Canton. Allele and genotype frequency of the eNOS gene was determined. Concerning the distribution of the respondents according to sex, 51 female respondents (51.0%) and 49 male respondents (49.0%) were analyzed in the total sample.

The respondents were divided into four age groups: ≤44, 45-65, 66-85 and ≥86. In the age group ≤44, 31 respondents (31.0%) were analyzed. In the age group 45-65 years, 54 respondents (54.0%) were analyzed. In the age group 66-86 years, 14 respondents (14.0%) were analyzed, and in the age group ≥86 year, one respondent (1.0%) was analyzed (Figure 1).

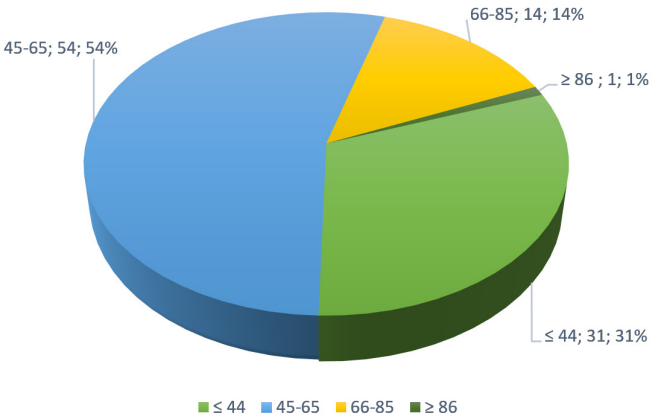

Figure 1. Distribution of respondents according to age group

Molecular genotyping of the eNOS 4a/b genotypes was detected by capillary electrophoresis on the ABI PRISM ®3500 genetic analyzer (Genetic Analyser 3500, Applied Biosystems).

We determined that the 390 bp fragment represents a polymorphic genotype (a/a, 4 repeats). Two fragments at 390 and 417 bp indicated a heterozygous genotype (a/b), and fragment at 417 bp that represents the wild-type genotype (b/b; 5 repeats) (Figure 2).

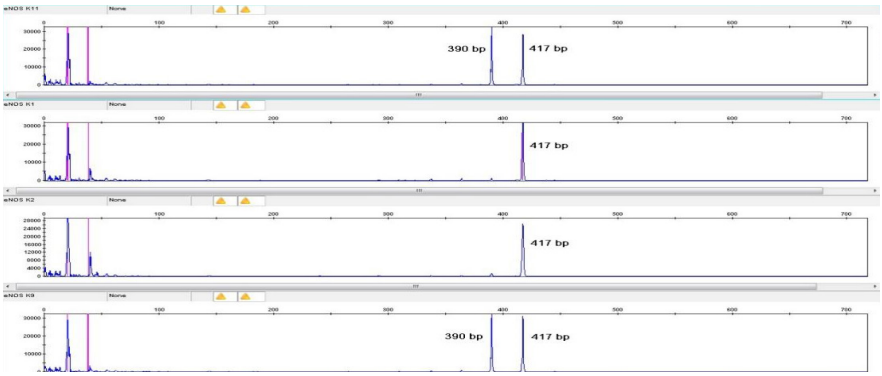

Figure 2. Electropherogram of eNOS gena (390 and 417 bp; 417 bp; 417 bp; 390 and 417 bp) detected by capillary electrophoresis on ABI PRISM ®3500 genetic analyser (Genetic Analyser 3500, Applied Biosystems)

The genotype distribution (absolute and relative genotype frequencies) of the eNOS gene (4a/b) was analyzed in 100 respondents of both sexes. Based on the results of molecular detection, there were 3 respondents of the (a/a) genotype, 24 respondents of the (a/b) genotype and 73 respondents of the (b/b) genotype. The analysis of allele and genotype distribution of the eNOS 4a/4b polymorphisms in subsamples according to sex indicated the highest frequency for the bb genotype, which was registered in 38 (38.0%) female respondents and 35 (35.0%) male respondents (Table 1).

Table 1. Absolute and relative genotype frequencies of eNOS gene according to sex

eNOS 4(a/b)		Sex		Total
		Female	Male	
Genotype (aa)	N (%)	1 (1.0%)	2 (2.0%)	3 (3.0%)
Genotype (ab)	N (%)	12 (12.0%)	12 (12.0%)	24 (24.0%)
Genotype (bb)	N (%)	38 (38.0%)	35 (35.0%)	73 (73.0)
Total	N (%)	51 (51.0%)	49 (49.0%)	100 (100.0%)

The analysis of statistical (χ^2) significance by comparing the presence of genotypes of the eNOS gene according to sex of the respondents did not show any significant differences in the distribution (χ^2 = .417; df = 2; p = .812; p > .05) (Table 1).

The allele frequency according to sex in the investigated loci was obtained by the determination (counting) of alleles from genotype values. The allele frequency (a) was 7.0% in the female respondents and 8.0% in the male respondents. The allele frequency (b) was 44.0% in the female respondents and 41.0% in the male respondents (Table 2).

Table 2. Absolute and relative allele frequencies of eNOS gene in subsamples according to sex

eNOS 4a/b	Sex		Total N=100 (100.0%)
	Female N=51 (51.0%)	Male N=49 (49.0%)	
Allele (a)	14 (7.0%)	16 (8.0%)	30 (15.0%)
Allele (b)	88 (44.0%)	82 (41.0%)	170 (85.0%)

The obtained results of differences in allele frequency of the eNOS gene according to sex of the respondents using a chi-square (χ^2) test did not indicate any significant differences in the distribution (χ2 = .436; df = 1; p = .607; p > .05).

The genotype distribution of the eNOS gene was also analyzed according to age groups of the respondents ($\leqslant$ 44, 45-56, 66-85 and $\geqslant$86) (Table 3 and 4).

Table 3. Absolute and relative genotype frequencies of eNOS gene according to age groups

eNOS 4a/b	Age group				Total
	≤44	45-65	66-85	≥86	
Genotype (aa)	1 (1.00%)	2 (2.00%)	0 (.00%)	0 (.00%)	3 (3.0%)
Genotype (ab)	7 (7.00%)	13 (13.00%)	4 (4.00%)	0 (.00%)	24 (24.0%)
Genotype (bb)	23 (23.00%)	39 (39.00%)	10 (10.00%)	1 (1.00%)	73 (73.0%)
Total	31 (31.00%)	54 (54.00%)	14 (14.00%)	1 (1.00%)	100 (100.0%)

In all age groups, the highest relative frequency was registered for the (b/b) genotype.

The absolute genotype frequency of the eNOS gene according to age groups and sex is shown in Table 4.

Table 4. Absolute genotype frequencies of eNOS gene according to age groups and sex

eNOS 4a/b	Age group								Total
	≤44		45-65		66-85		≥86		
	Female	Male	Female	Male	Female	Male	Female	Male	
Genotype (aa)	0	1	1	1	0	0	0	0	3
Genotype (ab)	1	6	9	4	2	2	0	0	24
Genotype (bb)	11	12	21	18	5	5	1	0	73
Total	12	19	31	23	7	7	1	0	100

Also, genotype frequency of the eNOS gene was analyzed according to sex, individually in each age group of the respondents. All age groups of the respondents according to sex indicate approximately the same frequencies for each genotype of the eNOS gene individually (Table 4).

DISCUSSION

In this study, the VNTR polymorphism of the eNOS gene was genotyped in the sample of 100 respondents from Tuzla Canton. In the total sample, 3.0% of the respondents of the (a/a) genotype, 24.0% of the respondents of the (a/b) genotype and 73.0% of the (b/b) genotype were determined. Endothelial nitric oxide synthase (eNOS) is essential for maintaining vascular homeostasis and has a vital role in modulating vascular endothelial function. Therefore, if a polymorphism can impact gene expression or protein structure of eNOS, it is likely that this polymorphism may lead to severe vascular endothelial dysfunction and influence predisposition to cardiovascular diseases (CVD) (Yu et al., 2020). Afef et al. (2016) analyzed the genotype presence of the eNOS gene in the Tunisian population, according to sex and age of the respondents. In the group of the female respondents with CVD, the genotype (a/a) frequency was 2.8%, and in the control group it was 7.6% OR (95% CI) = 7.19 (1.72-30.02). For the ab genotype in the group of the female respondents with CVD, the registered frequency was 69.8% and in the control group it was 26.1% OR (95% CI) = 1.1 (0.26-4.60). This study determined the frequency of 27.4% for the (b/b) genotype in the group of the female respondents with CVD, while in the control group it was 63.3% OR (95% CI) = 1. In the group of the male respondents with CVD the registered frequency for the (a/a) genotype was 2.1% and in the control group it was 4.5% OR (95% CI) = 3.54 (0.67-18.68). In the group of the male respondents with CVD, the frequency of the (a/b) genotype was 59.4% and it was 36.4% in the control group OR (95% CI) = 1.41 (0.27-7.35). The frequency of the (b/b) genotype in the group of the male respondents with CVD was 38.5% and in the control group it was 59.1% OR (95% CI) = 1.

Our study in the sample of 100 respondents from Tuzla Canton shows no statistically significant differences in the genotype distribution of the eNOS gene according to sex and age categories of the respondents ($p > .05$).

Table 5 gives a comparative representation of eNOS gene genotype frequencies in human population from Tuzla Canton with literature data.

Table 5. Comparative representation of genotype frequencies of eNOS gene in human population of Tuzla Canton with literature data

Population	Reference	Control group of respondents	Genotype frequency of eNOS gene (4a/4b)		
			aa	ab	bb
Sudan	Gamil et al., 2017	(N=78)	3 (3.8%)	25 (32.1%)	50 (64.1%)
Turkey	Sezgin et al., 2017	(N=61)	1 (1.6%)	14 (23.0%)	46 (75.4%)
Tunisia	Afef et al., 2016	(N=162)	10 (6.3%)	48 (30.4%)	100 (63.3%)
India	Narne et al., 2013	(N=121)	2 (1.6%)	42 (34.7%)	77 (63.6%)
Tunisia	Kallel et al., 2013	(N=225)	3 (1.3%)	58 (25.8%)	164 (72.9%)
Italy	Fatin et al., 2013	(N)=544)	14 (3.0%)	138 (25.0%)	392 (72.0%)
Turkey	Mehmetoğlu et al., 2010	(N=75)	1 (1.3%)	16 (21.33%)	58 (77.33%)
Turkey	Agirbasil et al., 2006	(N=100)	0 (.0%)	21 (21.0%)	79 (79.0%)
Slovenia	Milutinovic et al., 2005	(N=188)	10 (5.3%)	58 (30.9%)	120 (63.8%)
Tuzla Canton	Results of this research	(N=100)	3 (3.0%)	24 (24.0%)	73 (73.0%)

Comparison of our results with data from relevant literature sources has determined that polymorphism frequencies of the eNOS gene in the sample of healthy respondents from Tuzla Canton is in the scope of variations of values for this parameter with the literature data.

CONCLUSION

Preliminary results of the eNOS gene molecular genetic characterization in human population of Tuzla Canton as well as the Bosnian-Herzegovinian population are obtained by the realization of this study. The results represents a starting point for further research on the estimation of sensitivity and informativeness, especially in terms of the relating the eNOS gene polymorphisms with cardiovascular diseases. Testing new DNA polymorphisms as potential new markers in molecular diagnostics as well as analyzing and extending the number of the selected sample should continue in the future.

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