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The roles of endothelial nitric oxide synthase (eNOS) and myeloperoxidase (MPO) genes in microtia

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Abstract

Objective: The aim of this study was to determine the relationship between polymorphisms of endothelial nitric oxide synthase (eNOS) and myeloperoxidase (MPO) genes and development of microtia.

Methods: Nineteen (11 males, 8 females) unrelated cases with microtia and 40 healthy controls were enrolled in the present study. The study focused on three functional variants; a variant in exon 7 (G894T) and a variable number of 27 bp tandem repeats in intron 4 (VNTR) of eNOS gene and a variant in the promoter region (G463A) of MPO gene. We genotyped these variants using the polymerase chain reaction (PCR) and/or PCR-restriction fragment length polymorphism (RFLP) method. The distribution of allele and genotype in eNOS and MPO genes were compared between cases with microtia and healthy controls using chi-square test.

Results: With regard to the eNOS (G894T) variant, there was a significant difference in genotype distribution between cases with microtia and healthy controls (OR: 1.267, 95% CI: 1.004–1.598; p=0.009). Our study demonstrated that cases with eNOS (G894T) TT genotype had increased risk of microtia. The allele frequencies of eNOS (VNTR) variant showed statistically significant difference between cases with microtia and healthy controls (OR: 2.947, 95% CI: 1.188–7.311; p=0.028). eNOS (VNTR) B allele was higher in the cases. However, there was no significant difference for MPO (G463A) variant according to genotype distribution and allele frequency between cases with microtia and healthy controls.

Conclusion: To the best of our knowledge, this is the first analysis of the eNOS (G894T and VNTR) and MPO (G463A) variants in cases with microtia. Our data demonstrate that eNOS gene variants might play crucial role on the etiopathogenesis of microtia in Turkish population. The findings of the current study highlight the necessity for prospective longitudinal studies in elucidating the relative contributions of various factors in diseases with a multifactorial etiology where there is interplay among genetic susceptibility and exogenous factors.

Keywords: Microtia, endothelial nitric oxide synthase, myeloperoxidase, PCR, RFLP.

Özet: Endotelyal nitrik oksit sentaz (eNOS) ve miyeloperoksidaz (MPO) genlerin mikrotiyadaki rolü

Amaç: Bu çalışmanın amacı endotelyal nitrik oksit sentaz (eNOS) polimorfizmleriyle miyeloperoksidaz (MPO) genleri ve mikrotiya gelişimi arasındaki ilişkiyi belirlemekti.

Yöntem: Çalışmaya akraba olmayan 19 (11 erkek, 8 kadın) mikrotiyalı olgu ve 40 sağlıklı kontrol alındı. Çalışma, ekson 7'nin bir varyantı (G894T), eNOS geninin 4. nitronunda (VNTR) değişken sayıda 27 bp ardışık tekrarlar ve MPO geninin promoter bölgesinde (G463A) bir varyant olmak üzere üç fonksiyonel varyant üzerine odaklandı. Polimeraz zincir reaksiyonu (PCR) ve/veya PCR-restriksiyon parça uzunluk polimorfizm (RFLP) yöntemi kullanarak bu varyantların genotiplerini çıkardık. Ki-kare testi kullanarak mikrotiya olgularıyla sağlıklı kontroller arasında eNOS ve MPO genlerinde alel ve genotip dağılımını karşılaştırdık.

Bulgular: eNOS (G894T) varyantı açısından, mikrotiya olgularıyla sağlıklı kontroller arasında genotip dağılımı açısından önemli bir farklılık vardı (OR: 1.267, %95 GA: 1.004–1.598; p=0.009). Çalışmamız eNOS (G894T) TT genotipli olgularda mikrotiya riskinin arttığını gösterdi. eNOS (VNTR) varyanının alel sıklıkları mikrotiya olgularıyla sağlıklı kontroller arasında istatistiksel açıdan anlamlı farklılık olduğunu gösterdi (OR: 2.947, %95 GA: 1.188–7.311; p=0.028). Olgularda eNOS (VNTR) B aleli daha yüksek sıklıkta görülmüştür. Ancak genotip dağılımına göre MPO (G463A) varyantı, genotip dağılımı ve alel sıklığı açısından mikrotiya olguları ve sağlıklı kontroller arasında anlamlı bir farklılık yoktu.

Sonuç: Bildiğimiz kadarıyla bu çalışma ile mikrotiya olgularında eNOS (G894T ve VNTR) ve MPO (G463A) varyantları ilk kez incelenmiştir. Verilerimiz eNOS gen varyantları Türk halkındaki mikrotiyanın etyopatogenezinde kritik rol oynayabildiğini göstermektedir. Güncel çalışmanın bulguları genetik yatkınlık ve dışsal etmenlerin etkileşimde olduğu bir multifaktöryel etiyolojili hastalıkta değişik faktörlerin göreceli katkılarını aydınlatmada prospektif uzunlamasına çalışmaların gerekliliğini vurgulamaktadır.

Anahtar sözcükler: Mikrotiya, endotelyal nitrik oksit sentaz, miyeloperoksidaz, PCR, RFLP.

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Microtia is a congenital deformity affecting the outer ear, characterized by a small, abnormally shaped auricle. External ear canal is commonly narrowed, blocked or absent and middle ear is underdeveloped because the outer ear and the middle ear have common embryologic origin.[1] The prevalence of microtia varies between 0.83 and 17.4 per 10,000 births.[2] It is also reported that microtia is more common in males, and most cases are unilateral, predominantly being on the right side.[3] The pathogenesis of microtia remains unclear. Several risk factors including prenatal exposure to drugs, paternal age, high parity, maternal diabetes, high maternal age, multiple births have been implicated with this deformity.[2–5] The hereditary factors are most likely associated with microtia because it is generally seen some specific syndromes with chromosomal abnormalities, including Goldenhar syndrome, Treacher Collins syndrome, trisomy 21 and trisomy 18.[3]

Nitric oxide (NO) acts as an essential molecular mediator in many physiologic processes that are important for organogenesis, such as gene expression, cell growth, matrix remolding, proliferation, differentiation and apoptosis.[6] In embryonic tissues, the expression of NO sythase isoforms is temporally and spatially regulated, and impairment of endogenous NO secretion can result in developmental defects. The catalyst in endothelial NO synthesis is endothelial nitric oxide synthase (eNOS). A functional variant in exon 7 of human eNOS is related to a Glu-Asp change at codon 298 (Glu298Asp, also called G894T) (rs1799983). The GG ancestral genotype of the eNOS G894T variant, located in exon 7 of the eNOS on chromosome 7, has been claimed to cause increased protein expression and activity.[7] Other functional variant is a variable number of tandem repeats (VNTR, 27 nt) in intron 4, which accounts for >25% of basal plasma NO production.

Myeloperoxidase (MPO) is a lysosomal hemoprotein enzyme related with oxidative stress, located in polymorphonuclear neutrophils and monocytes. This enzyme catalyzes production of hypochloric acid (HOCl), which in turn may lead to damage in host DNA and result in the mutation of homeobox, oncogenes and tumor supressor genes.[8] The MPO gene has a common variant within the gene promoter. This guanin 463 adenine (G463A) (rs2333227) base transition has been described at the SP1 binding site, where the variant A allele is linked with reduced messenger RNA (mRNA) expression, leading to approximately 25 times less transcription activity compared to the G allele.[9]

In this study, we examined the relationship between eNOS (G894T and VNTR), MPO (G463A) gene variants and microtia risk.

Materials and Methods

Study population

Nineteen (11 males, 8 females) nonsyndromic, unrelated cases with microtia and 40 healthy controls were examined in the study. We genotyped the eNOS (G894T and VNTR) and MPO (G463A) variants. Informed consent was obtained from each participant before blood sampling, and the study was approved by the local Ethical Committee.

Genotyping procedure

DNA isolation: Peripheral blood samples were collected from the cases with microtia and healthy controls. Genomic DNA was extracted from EDTA (ethylenediamine tetraacetate)-treated peripheral venous blood using salting out method and stored at -20 °C until analysis.[10]

eNOS (G894T) variant genotyping: The eNOS variant was analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) assays. The segment amplification of exon 7 with the flanking intronic primers 5'-CATGAGGCTCAGCCCCAGAAC-3' (sense) and 5'-AGTCAATCCCTTTGGTGCTCAC-3' (antisense) followed by MboI restriction endonuclease (Invitrogen CA, USA) digestion for 16 hours at 37 °C. Digestion was resolved on 3% agarose gel and visualized using ultraviolet light. The 206 bp PCR products had a consistent restriction site resulting in 119 bp and 87 bp fragments. Twenty percent of the samples were duplicated as internal quality control to avoid sample or reading errors.[11]

eNOS (VNTR) variant genotyping: eNOS intron 4 variant was analyzed by PCR using following primer: F: 5'-AGGCCCTATGGTAGTGCCTTT-3', and R: 5'-TCTCTTAGTGCTGTGGTCAC-3'. The PCR product (393 bp and/or 420 bp) was obtained. The products were then separated on 4% NuSieve GTG agarose gel. The experimental process was repeated twice for each sample.[12]

MPO (G463A) variant genotyping: The region with G463A variant which is located at promoter of MPO gene was multiplied with PCR by using MPO-F 5'-CGG TAT AGG CAC AAT GGT GAG and R: 5' GCA ATG GTT CAA GCG ATT CTT C primary chains and amplification control was done with 2% agarose gel electrophoresis. Amplified region was incubated for 16 hours with 5 units of *AciI* enzyme at 37 °C and analyzed with 3% agarose gel electrophoresis.[13]

Statistical analysis

All data were analyzed using software SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL, USA). The statistically significant differences between cases with microtia and healthy controls were estimated by logistic regression analysis. Odds ratio (OR) and 95% confidence interval (CI) were also calculated. The differences in eNOS (G894T and VNTR) and MPO (G463A) variants genotype frequencies between cases with microtia and healthy controls were compared with chi-square test, and Fisher's exact test was used when needed (http://ihg.gsf.de/cgi-bin/hw/hwa2.pl). All analyses were two-tailed, and differences were interpreted as statistically significant when <0.05.

Results

The genotype and allele distributions of the eNOS (G894T and VNTR) and MPO (G463A) variants were presented in Table 1.

eNOS (G894T) variant: The distribution of GG, GT and TT genotypes for eNOS (G894T) were observed in 65%, 35%, 0% of healthy controls and in 52.6%, 26.3% and 21.1% of cases with microtia, respectively. The allele frequency of G and T were 82.5% and 17.5% in healthy controls, and 65.8% and 34.2% in cases with microtia. A statistically significant difference between eNOS variant and microtia was determined (OR: 1.267, 95% CI: 1.004–1.598; p=0.009). The cases with eNOS TT genotype had increased risk of microtia.

eNOS (VNTR) variant: The distribution of AA, AB and BB genotypes for eNOS3 (VNTR) variant were observed in 72.5%, 25% and 2.5% of healthy controls and in 47.4 %, 36.8% and 15.8% of cases with microtia, respectively. While the allele frequency of A and B were 85%, 15% in healthy controls and 65.8%, 34.2% in cases with microtia. The allele frequencies of eNOS variant showed statistically significant difference between cases with microtia and healthy controls (OR: 2.947, 95% CI: 1.188–7.311; p=0.028). eNOS B allele was higher in cases with microtia.

MPO (G463A) variant: The distribution of GG, GA and AA genotypes for MPO (G463A) variant were 65%, 30% and 5% in healthy controls and 47.4%, 42.1% and 10.5% in cases with microtia, respectively. We were unable to determine statistically significant difference in any genotype or allele frequency of MPO when cases with microtia and healthy controls were compared.

Discussion

Nitric oxide modulates the growth of smooth muscle and regulates blood flow through smooth muscle cells, decreases endothelial permeability and influences leukocyte adhe-

Table 1. The genotype distribution and allele frequencies of the eNOS (G894T), (VNTR) and MPO (G463A) variants in cases with microtia and healthy controls.*

	Genotype/Allele	Patients n† (%)	Controls n§ (%)	OR (95% CI)	p
eNOS (G894T)	GG	10 (52.6)	26 (65)	0.598 (0.197–1.816)	0.403
	GT	5 (26.3)	14 (35)	0.633 (0.198–2.225)	0.565
	TT	4 (21.1)	0 (0)	1.267 (1.004–1.598)	**0.009**
	G allele	25 (65.8)	66 (82.5)		
	T allele	13 (34.2)	14 (17.5)	2.451 (1.013–5.935)	0.060
eNOS (VNTR)	AA	9 (47.4)	29 (72.5)	0.341 (0.110–1.064)	0.083
	AB	7 (36.8)	10 (25)	1.750 (0.540–5.668)	0.372
	BB	3 (15.8)	1 (2.5)	7.313 (0.707–75.669)	0.094
	A allele	25 (65.8)	68 (85)		
	B allele	13 (34.2)	12 (15)	2.947 (1.188–7.311)	**0.028**
MPO (G463A)	GG	9 (47.4)	26 (65)	0.485 (0.160–1.471)	0.260
	GA	8 (42.1)	12 (30)	1.697 (0.546–5.276)	0.390
	AA	2 (10.5)	2 (5)	2.235 (0.290–17.220)	0.588
	G allele	26 (68.4)	64 (80)		
	A allelle	12 (31.6)	16 (20)	1.846 (0.769–4.435)	0.174

*Fisher's exact test. †n=19, §n=40

sion to vascular endothelium.[14] The impairments in NO production promote thrombogenesis through platelet adhesion and aggregation, and production of cytokines and adhesion molecules.[15] During embryonic growth, the cell numbers were determined by the balance between cell proliferation, differentiation, migration and apoptosis. Nitric oxide acts on a variety of physiological and pathological pathways such as the regulation of the balance between apoptosis and mitosis, and have an inhibitory on cell proliferation.[16] In embryogenesis of Drosophila, it regulates the balance between cell proliferation and differentiation.[17] The deficiency of NO generation leads to endothelial dysfunction, which in turn facilitates the development of several disorders such as type II diabetes mellitus, insulin resistance, and cardiovascular events. NO is produced from L-arginine by 3 nitric oxide synthase isoenzymes and eNOS gene is one of them. eNOS is mainly produced by vascular endothelial cells and has a key role in the modulation of vascular tonus and angiogenesis. It is also expressed in several cell types, including bronciholar and renal epithelial cells, cardiomyocytes, and neutrophils.[18] The relations of eNOS with the actin cytoskeleton, microtubules, and intermediate filaments were studied with great interest.[18]

The changes in NO generation caused by cytoskeletal reorganization play a significant role in numerous physiological and pathophysiological conditions. The G894T variant located in exon 7 causes an amino acid substitution at position 298 (Glu298Asp) which may result in proteolytic cleavage of the eNOS protein and may diminish NO bioavailability, rather than altering generation of NO, in subjects with the GT and TT genotypes compared to those with GG genotype in a dose-dependent manner.[19] VNTR variant of eNOS gene is associated with plasma concentrations of NO.[20] In repeats of a 27-bp consensus sequence, two alleles, a common large allele and a smaller allele, were found. It is noteworthy that the larger allele, designated "b-insertion", has five tandem repeats, and the smaller allele "a-deletion" has four repeats.

Both endogenous processes and exogenous exposures are likely to generate reactive oxygen species (ROS). Reactive oxygen species may cause oxidative damage to DNA and other macromolecules, thereby leading to genetic alterations, a process modulated by several antioxidant systems which may change the balance between prooxidant cellular activity and antioxidant defense system.[21] Reactive oxygen species act as primary or secondary messengers in processes related to cellular growth or death. A variety of examples demonstrate the crucial role of ROS in development as redox status is one of the major regulator of the basic transcription factors that affect cell signaling pathways related to proliferation, differentiation, and apoptosis. Thus, oxidative stress may modify several reactions that have an impact on embryonic development both positively and/or negatively.[22] MPO produces ROS endogenously by behaving like an antimicrobial enzyme, catalyzing hydrogen peroxide-dependent oxidation of chloride to generate a strong oxidizing agent, HOCl. HOCl contributes to generation of secondary oxidation products by reacting with other biological molecules.[21] The variant of MPO G463A within the MPO-463 gene promoter has been studied extensively and an association between high activity of G463A G allele and increased MPO activity was reported for various diseases. The lower activity A allele, which is associated with lower levels of polycyclic aromatic hyrocarbons and ROS production, implicated lower risk for relevant diseases.[23]

In the current study, the distribution of the eNOS (G894T and VNTR), MPO (G463A) genotypes between the cases with microtia and healthy controls were evaluated. We found that eNOS (G894T) and (VNTR) variants were statistically different between these two groups. The cases with eNOS (+894) TT genotype had increased risk of microtia (p=0.009) (Table 1). Also, eNOS (VNTR) B allele was higher in the cases (p=0.028) (Table 1). However, there was no significant difference for MPO (G463A) variant according to genotype distribution and allele frequency between the cases with microtia and healthy controls.

Conclusions

To the best of our knowledge, this is the first study in which the relationship between eNOS, MPO gene variants and microtia was evaluated. Our data suggest that eNOS gene variants may play a role in the etiopathogenesis of microtia in Turkish population. Although etiopathology of microtia is still unclear, we thought that genetic variations might influence development of embryologic phase. Therefore, prospective longitudinal studies, mainly focusing on to reveal the contributions of genetic susceptibility and exogenous factors in microtia is required.

Conflict of Interest: No conflicts declared.

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