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Evaluation of the methylation status of the MB-COMT, APC2, NR3C1, and DRD2 genes in Turkish patients with microtia

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ABSTRACT

Aims: Microtia is defined as a congenital malformation of the middle and external ears. DNA methylation is the major epigenetic modification of genomic DNA that is regulated in the early embryonic stage. In this study, we analyzed the methylation status of the MB-COMT, APC2, NR3C1, and DRD2 genes in patients with microtia.

Methods: The blood samples were taken from microtia patients and healthy controls. Genomic DNA was isolated using a commercial kit. The methylation status of the MB-COMT, APC2, NR3C1, and DRD2 genes was analyzed using the methylation-specific polymerase chain reaction (MS-PCR) method. The results were evaluated statistically.

Results: The DRD2 methylation status was found to be associated with microtia ($p<0.001$). We found that the DRD2 gene was partially methylated in all patients with microtia. There was no significant difference between the methylation status of the MB-COMT, APC2, and NR3C1 genes and microtia.

Conclusion: To our knowledge, this is the first study in our country to evaluate the relationship between the methylation of these genes and the risk of microtia. Our results demonstrate the presence of epigenetic changes in the DRD2 gene during microtia development. Methylation may have contributed to the pathogenesis of microtia as it affects gene expression. Studies with larger sample sizes and in different ethnic groups are needed to further investigate the role of these genes in microtia.

Keywords: Microtia, genes, methylation

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INTRODUCTION

Microtia is defined as a congenital malformation of the middle and external ears. Microtia occurs when the first sulcus and the first and second arches in the embryonic stage are abnormally developed. Microtia may be characterized by facial and mandibular soft tissue dysplasia and hearing loss.[1] It may be bilateral or unilateral, and there may be auricular deformities ranging from mild structural abnormalities to complete ear loss. Pregnancy issues and parental status, such as education level and age of the mother's diabetes, taking medicine, multiple births, drinking during pregnancy, smoking, or an early pregnancy infection, significantly affect the incidence rate, according to the risk factor analysis. The risk of microtia may also increase due to the lack of sufficient folic acid intake during early pregnancy.[2] As a multifactorial disease, microtia may involve genetic and environmental factors and interactions between them. Researchers have attempted to detect the genetic factors affecting microtia development and the related action mechanisms. However, there is no general consensus on the mechanism of microtia.

In DNA methylation, the expression of genes in eukaryotic cells is regulated in a common epigenetic way, and genomic DNA undergoes significant epigenetic change.[3] DNA methylation affects the gene expression process and can be inherited through meiosis and mitosis.[4] Catechol-O-Methyltransferase (COMT) is primarily involved in the metabolism of estrogen and dopamine. Two different protein isoforms, i.e., S-COMT (soluble isoform), and MB-COMT (membrane-bound isoform), each with its own promoter, are encoded by the human COMT gene on chromosome 22q11.21.[5] In monozygotic twins discordant for low birth weight, the COMT gene region exhibited differential DNA methylation, which shows the DNA methylation mechanism based on which the schizophrenia risk is affected by birth weight.[6] Different biological diseases and processes require the canonical WNT signaling pathway.[7] COMT converts 2-hydroxy estradiol to 2-methoxy estradiol (2ME), a compound with biological functions involved in regulatory processes associated with pregnancy and placentation. COMT and 2ME play a role in regulating angiogenesis, trophoblast development, and hypoxia

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adaptation.[8] COMT-deficient mouse models have interrogated the molecular mechanisms underlying preeclampsia and fetal growth restriction.[8] The ability of the adenomatosis polyposis coli homologue (APC2) to regulate the beta-catenin/WNT signaling pathway has been demonstrated in Drosophila and cancer cell lines.[9] When the rat brain is developed, APC shows that the expression pattern is dramatically alternated and expressed exclusively in postmitotic neuron.[10] As a biological consequence of excessive Wnt signaling in vertebrates, a secondary axis is formed in the embryo.[11] Glucocorticoids (GCs), the steroid hormones that are produced mainly by the adrenal glands, mediate stress responses. Pharmacologically, GC excess has an impact on stress biology. It balances homeostatic effects that facilitate survival and short-term recovery from hardship, while its acute and chronic effects are well defined.[12] GCs also affect normal fetal development. It ensures the development and maturation of different fetal tissues, such as skeletal muscle, the intestine, the liver, the lungs, and adipose tissue.[13] The nuclear receptor subfamily 3 group C member 1 (NR3C1) gene encodes the glucocorticoid receptor. Schmidt et al. reported that NR3C1 affects DNA methylation at birth, supporting the hypothesis that DNA methylation in the placenta correlates with adult frontal cortex DNA methylation and anxiety-like phenotypes.[14] Nr3C1 is located on chromosome 5 and contains 17 exons. Nine exons are non-coding and are placed at gene promoter.[15] Dopamine signaling critically contributes to adult brain function and neural development, such as locomotion, reward, memory, and learning.[16] There are two types of dopamine receptors in the brain based on sequence homology and function: inhibitory D2-like receptors (D3, D2, and D4) and excitatory D1-like receptors (D1 and D5). Although all dopamine receptors are important for brain homeostasis, dopamine receptor D2 (DRD2) has a close relationship with brain disorders.[17]Adult D2 expression is significantly affected by the interaction of DRD2 single nucleotide polymorphisms (SNPs) and early developmental factors.[18] This may explain why early life adversities have important individual differences in dopamine-related processes and disorder susceptibilities.[19] The human DRD2 gene on chromosome 11q22-23 is arranged in eight exons that span at least 270 kilobases.[19] In our previous study, we found partial methylation of the DRD2 gene in patients with schizophrenia.[20] In light of this information, we aimed to evaluate the methylation status of the MB-COMT, APC2, NR3C1, and DRD2 genes, which play a role in embryonic development in microtia patients.

METHODS

Study Population

The study population included 102 unrelated healthy control subjects and 18 subjects with microtia. The subjects with microtia were selected from those followed up and treated in the Department of Plastic, Reconstructive and Aesthetic Surgery, School of Medicine, Kahramanmaraş Sütçü İmam University, Kahramanmaras. The study was approved by the Medical Ethics Commıttee of Medical Faculty, University of Gaziantep University (Date: 19.02.2009, Decision no: 02-2009/23). Cases with syndromic findings in addition to microtia were not included in the study. The patients living in the same geographical areas were chosen as the healthy group, and they were well matched with the patient group in gender and age. The controls did not have a family or personal history of dysmorphic disorders. All patients, subjects, and controls were of Turkish origin. All subjects submitted informed written consent before enrollment in the study, based on the ethical guidelines of the 2008 Declaration of Helsinki. The local human research ethics committee approved the study.

Methylation-Specific Polymerase Chain Reaction (MS-PCR)

The blood samples were taken from all subjects and placed in 2-ml ethylenediaminetetraacetic acid (EDTA) tubes. The commercial kit was used to extract the genomic DNA (Plus Blood Genomic Purification Kit, Genemark, USA). Then, an EZ-96 DNA methylation-gold kit was used for bisulfite conversion of genomic DNA (Orange, Zymo Research, CA). The methylation status of the MB-COMT, APC2, NR3C1, and DRD2 genes was analyzed with the methylation-specific polymerase chain reaction (MS-PCR) method previously described.[21-24]

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows (SPSS, Inc., Chicago, IL). The means and standard deviations were used for the presentation of continuous quantitative variables. The methylation status of these genes was determined using the Fisher exact test and the Monte Carlo chi-squared test. A p-value smaller than 0.05 was considered statistically significant.

RESULTS

MB-COMT, APC2, NR3C1, and DRD2 gene promoter methylation were evaluated in a total of 120 samples (18 microtia and 102 healthy controls). The demographic characteristics of the participants are shown in **Table 1**.

Table 1. Demographic characteristics of all participants

	Patients n=18 (%)	Controls n=102 (%)
Age (mean, years)	11.44	22.41
Gender		
Female	8 (44.44)	31 (30.39)
Male	10 (55.56)	71 (69.61)

It was found that the DRD2 gene was partially methylated in patients with microtia ($p<0.001$). The DRD2 gene partial methylation was higher in patients with microtia than in healthy controls. The methylation status of the NR3C1, APC2, and MB-COMT genes and microtia did not differ significantly. The methylation status of these genes is summarized in **Table 2**.

Table 2. Methylation status of *MB-COMT, APC2, NR3C1*, and *DRD2* genes in groups

Genes	Methylation status	Patients n:18 (%)	Controls n:102 (%)	OR Exp (B)	95% CI	p*
DRD2	Unmethylated	0 (0)	43 (42.2)	1.729*	1.465-2.040*	0.001*
	Partial methylated	18 (100)	59 (57.8)			
COMT	Unmethylated	1 (5.6)	14 (13.7)	2.705*	0.333-21.957*	0.464*
	Partial methylated	17 (94.4)	88 (86.3)			
NR3C1	Unmethylated	0 (0)	9 (8.8)	1.097*	1.033-1.165*	0.352*
	Partial methylated	18 (100)	93 (91.2)			
APC2	Unmethylated	18 (100)	94 (92.2)	1.085*	1.025-1.148*	0.604*
	Partial methylated	0 (0)	8 (7.8)			

*: Fisher's Exact Test.

DISCUSSION

Microtia includes congenital anomalies of the auricle, which can range from mild structural abnormalities of the ear to loss of the ear (anotia).[25] Microtia is a public health problem that causes psychosocial sequelae, in part because of the burden of having multiple surgeries and the stigma associated with ear malformations. Studies on the prevalence of microtia in France, Italy, the United States, Sweden, and Finland have shown that the prevalence rate varies between 0.83 and 4.34 per 10,000 births.[26] This topic has been studied with different genetic techniques such as DNA sequencing, single gene disorders, linkage analysis, cytogenetic rearrangements, and animal models. There is evidence of a significant genetic influence in the microtia origin: 1) familial cases with recessive or autosomal dominant modes of inheritance with incomplete penetrance and variable expression;[27] 2) monozygotic twin concordance higher than dizygotic twin concordance; 38.5% and 4.5%, respectively;[28] 3) familial case estimates ranging between 3 and 34%;[29] 4) over 18 different syndromes related to microtia with reported chromosomal aberrations or single-gene defects, and 5) mouse models that demonstrate that specific gene mutations cause microtia.

DNA methylation is important as a mediating mechanism of gene expression. In addition, it is an epigenetic modification involved in many physiological processes such as aging, X-chromosome inactivation, transposable element silencing, and genomic imprinting.[30] DNA methylation in mammals occurs primarily on the cytosine-guanine dinucleotides' cytosines, called CpG islands. There is considerable evidence showing the susceptibility of epigenetic programming to the early embryonic environment, such as toxin exposures and nutrient availability before implantation of the blastocyst. These environmental cues leading to growth defects due to changes in gene expression have disrupted the embryonic wave dynamics of DNA methylation-proper erasure, methylation maintenance, and de novo methylation-crucial for the developmental program. Methylation of DNA impacts the gene expression process and can persist and be inherited with meiosis and mitosis in cells 4. In the study, the craniofacial tissue of a 9.5-day-old fetal rat was found to show DNA methyltransferase positive expression, showing that methylation of DNA is regulated in the embryo's early phase.[31] The Sox4 gene, a strong candidate gene for cleft lip and palate, is an essential regulatory gene for developing and differentiating neural crest cells during embryonic development.[32] The impacts of the environment on embryonic development using in vitro models have been extensively studied. However, there is little study on the embryonic environment's direct epigenetic impacts and the long-term effects on long-term development because there are a limited number of cells and some technological barriers during the stages before implantation.[33] A systematic review of 173,687 children with deformities and 11.7 million controls showed an association between smoking during pregnancy and various neonatal defects, such as microtia.[34] The study also showed that undesirable environmental factors, including alcohol and tobacco, induce abnormal methylation of the associated susceptibility genes at the time of craniofacial morphogenesis and cause craniofacial morphological abnormalities.[35,36]

This study aimed to investigate the methylation status in patients with microtia (MB-COMT, APC2, NR3C1, and DRD2). This study is the first to study the association between these genes' methylation and microtia risk in our country, as far as we know. When DRD2 methylation status was compared between patients and controls, partial DRD2 methylation was found in all patients with microtia (**Table 2**). The methylation status of other genes was similar between patients and controls. Gene expression is affected by DNA methylation. There are studies showing that DRD2 gene methylation is associated with Parkinson disease, schizophrenia, and cannabinoid or synthetic cannabinoid use disorders.[37-39] In a study, it was shown that DRD2 methylation is negatively associated with regional brain gray matter volumes in children from families at high risk for alcohol dependence.[40] There are no studies examining the relationship between microtia and DRD2 gene methylation.

This analysis has several limitations. The first limitation was the small sample size, which could limit statistical power. The second limitation is that it evaluated only four genes' methylation status. Other genes may also affect the development of the microtia. Finally, covariates that could affect DNA methylation status were not examined. The strength of our study is that it is the first time to evaluate MB-COMT, APC2, NR3C1, and DRD2 methylation status in patients with microtia in the Turkish population.

CONCLUSION

Our results showed that the DRD2 gene is methylated in patients with microtia. Since this methylation affects gene expression, it may have played a role in the pathogenesis of the malformation. A larger sample size and different ethnicities are required to further evaluate this association.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was approved by the Medical Ethics Committee of Medical Faculty University of Gaziantep University (Date: 19.02.2009, Decision No: 02-2009/23).

Informed Consent: Written informed consent was obtained from subjects and patients who participated in this study

Referee Evaluation Process: Externally peer-reviewed.

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