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Investigation of leukocyte telomere length and hTERT gene MNS16A VNTR variant in microtia patients

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ABSTRACT

Aim: Telomeres are the basis of replicative senescence in somatic cells and control cell division. It has been shown in some studies that telomere shortening is associated with growth retardation and congenital malformations. Microtia is acongenital ear deformity in which the external ear is malformed and underdeveloped. This study aimed to determine whether leukocyte telomere length (LTL) and the MNS16A Variable Number Tandem Repeat (VNTR) variant of the hTERT gene are associated with the risk of microtia in the Turkish population.

Methods: A total of 38 volunteers, 18 patients diagnosed with microtia and 20 healthy controls, were included in the study. LTL analysis was performed with the Quantitative PCR method, and relative T/S ratios of patients and controls were calculated. hTERT-MNS16A-VNTR analysis was performed by PCR method and analysed agarose gel electrophoresis.

Results: When patients and healthy controls were compared in terms of genotype/allele frequencies; no statistically significant difference was detected in the genotype and allele frequency of the hTERT-MNS16A-VNTR variant. However, when the T/S ratios of the patients were compared with the healthy group, borderline significance was detected in terms of the shortening rate (p=0.055).

Conclusions: Our study is the first study in the literature to examine the relationship between microtia and LTL and hTERT-MNS16A-VNTR. The results suggest that the hTERT-MNS16A-VNTR variant may not be associated with microtia, but telomere shortening may have a causal relationship with microtia. Since microtia is a rare congenital anomaly with varying prevalence among populations, studies in different ethnicities and with larger sample groups will further elucidate the relationship between microtia and LTL/hTERT-MNS16A-VNTR.

Keywords: Microtia, leukocyte telomere length, VNTR, PCR, DNA.

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1. Introduction

Microtia is a congenital malformation that occurs in varying severities, ranging from mild structural abnormalities to absence of the outer and middle ear [1]. Microtia occurs in 1 to 10 births in 10,000, and the risk of microtia is

estimated to be 20-40% higher in men than in women. Although 77-93% of affected individuals have unilateral involvement, microtia can also occur bilaterally [2,3]. Microtia is a multifactorial disease in which environmental and genetic factors or interactions between the two may be involved [4]. In addition to ethnicity and male gender, microtia risk factors include low birth weight and maternal diseases such as diabetes [3]. Ninety percent of microtia cases are unilateral, and bilateral microtia cases indicate a higher risk of other associated anomalies or an underlying syndrome [5]. Although there has been significant progress in understanding the etiology of microtia, the specific pathophysiology mechanism has not yet been fully elucidated [6].

Telomeres are nucleoprotein structures located at the end of each chromosome arm and provide genome stability. It consists of a hexameric (TTAGGG) tandem repeat DNA sequence that is highly conserved in all mammals [7]. It is known that telomeres play an important role in the cellular aging process [8]. Epidemiological data show an association between structural telomere length and various diseases, including cancers. Several single nucleotide polymorphisms at different loci identified by genome-wide association studies affect interindividual variation in telomere length [9]. Some studies have shown that telomere shortening is also associated with growth retardation and congenital malformations [10]. The human telomerase reverse transcriptase gene (*hTERT*), which is responsible for telomere synthesis, is located in the 5p15.33 chromosome region and encodes a ribonucleoprotein enzyme that lengthens the chromosome ends that shorten with each cell division [11]. The *hTERT* gene covers a 40 kb DNA region consisting of 16 exons and 15 introns. This gene produces an 1132 amino acid long polypeptide, which is then translated into the 130 kD functional TERT protein [12]. The MNS16A gene variant is located in exon 16 of the *hTERT* gene and consists of 23 bp core tandem repeats separated by the CAT trinucleotide, which harbors a transcription factor binding site [13]. MNS16A variants according to variable number tandem repeat (VNTR) allele lengths have been studied in different types of cancer, neurodegenerative diseases, and metabolic diseases, as well as in other populations. While the hTERT-MNS16A-VNTR S allele is associated with bladder cancer risk in the Indian population [14], the hTERT-MNS16A-VNTR LL genotype in Alzheimer's disease in the Italian population is associated with an increased risk of Alzheimer's disease only in men, and the hTERT-MNS16A-VNTR L allele has been shown to reduce *hTERT* expression [15].

This study aimed to determine whether leukocyte telomere length (LTL) and the MNS16A VNTR variant of the *hTERT* gene are associated with the risk of microtia in the Turkish population.

2. Materials and methods

A total of 38 volunteers, 18 patients, 9 female and 9 male, diagnosed with microtia, and 20 healthy controls were included in the study. The patient group was selected from those treated and followed up in the Department of Plastic, Reconstructive, and Aesthetic Surgery. All individuals in the patient and control groups were of Turkish origin. The healthy control group is balanced in terms of age and gender. The healthy control group was selected from patients living in the same geographical regions. The average age of the patient group was 11.44 and the control group was 13.45. Genomic DNA was isolated from blood leukocytes collected in EDTA tubes, after obtaining consent from the individuals, with a commercial kit (ELK Biotech DNA isolation kit) by the manufacturer's instructions.

For LTL analysis of the obtained DNAs, a dilution of 0.5 to 2 ng/µl was made in the DNA sample of each individual. LTL analysis was performed by qPCR method using ELK Biotechnology Human Telomere Length Quantification qPCR Assay Kit (Relative) (Cat No: EQ022-01). Two qPCR reactions were prepared for each sample: telomere sequence copy number and reference gene single copy number (SCR). SCR primers specifically recognize the 78 bp long region on human chromosome 11. T/S ratios were calculated using the telomere sequence copy number (T) and reference gene single copy number (S) values in each patient's sample with the $2^{-\Delta\Delta Ct}$ formulation. Relative T/S ratios of patients and controls were calculated for each individual [16].

hTERT-MNS16A-VNTR analysis was performed by PCR method. DNA samples were amplified under appropriate PCR conditions using primers appropriate for the region to be analyzed. Then, the samples were carried out in agarose gel electrophoresis (3.5%) and genotyped under UV light. The long (L) allele showed bands with lengths of 302 bp and 332 bp, and the short (S) allele showed bands with lengths of 243 bp and 272 bp [15].

Statistical analyses were performed with SPSS package software (IBM Corp., Armonk, NY, USA). The Pearson chi-square test or Fisher's exact test, was used to compare the discrete variables. A p-value of less than 0.05 was accepted as significant. Genotype distributions of the studied variant were analyzed in terms of Hardy-Weinberg equilibrium (HWE) and by chi-square tests.

3. Results

When the T/S ratios of the patients were compared with the healthy group, borderline significance was found in terms of the shortening rate (p = 0.055) (Table 1).

Table 1. Comparison of T/S ratio between microtia patients and healthy controls.

Parameters	**Microtia** (n= 18)	**Healthy Control (**n=20)	***P****
Telomere T/S Ratio	2629 ±2044	3599 ± 1456	0.05[#]

*Values: Mean ±SD; * Pearson Chi-Square Tests, # Independent-Samples Mann-Whitney U Test.*

When patients and healthy controls were compared in terms of genotype/allele frequencies; No statistically significant difference was detected in the genotype and allele frequency of the hTERT-MNS16A-VNTR variant. There is no deviation from HWE for this variant in both the patient and healthy control groups (Table 2). An example gel result of the genotype analysis of the hTERT-MNS16A-VNTR variant is shown in Figure 1.

Table 2. Comparison of genotype and allele frequencies of hTERT-MNS16A-VNTR traits between microtia products and healthy controls.

Variables	**Geno -type**	**Microtia**	**Healthy Control**	***P****
hTERT VNTR		n= 18 (%)	n= 20 (%)	
Genotype	LL	5 (%28)	8 (%40)	0.726
	LS	10 (%55)	9 (%45)	
	SS	3 (%17)	3 (%15)	
Allele	L	20 (%56)	25 (%62,5)	
	S	16 (%44)	15 (%37,5)	0,538
HWEp		0,595883	0,858028	

** Pearson Chi-Square Tests.*

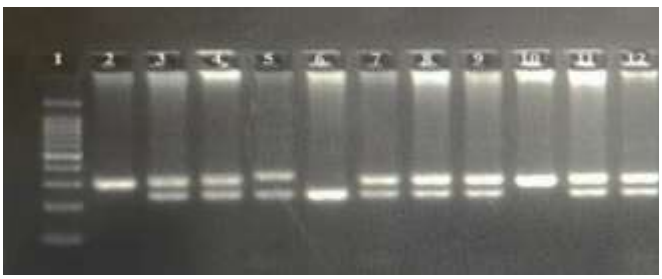

Figure 1. Genotype analysis of hTERT-MNS16A-VNTR variant agarose gel images.
(1: DNA marker (100 bp); LL:2,10; LS:3,4,5,7,8,9,11,12 ve SS:6).

4. Discussion and Conclusions

In our study, we performed analyses of LTL and hTERT-MNS16A-VNTR variant in microtia patients and healthy control groups. When the hTERT-MNS16A-VNTR variant was compared with patients and healthy controls in terms of genotype/allele frequencies; It was determined that there was no statistically significant difference in genotype and allele frequency. However, when the T/S ratios of LTL were compared with the healthy group in terms of telomere shortening in the patient group, it was found to be borderline significant in terms of telomere shortening rate (p=0.055). As a result of the literature review, it was seen that these analyses were performed for the first time in microtia patients.

In a study investigating telomere length in the prenatal period, 282 samples, 217 of which were obtained from amniotic fluid and 65 of which were obtained from human chorionic villi, were included in the study. In chorionic villus samples, telomeres of fetuses with a single congenital anomaly were found to be significantly shorter than controls, while when looking at a single anomaly type, no significant difference was found in terms of telomere length between fetuses with isolated heart malformation or diaphragmatic hernia and controls. In amniotic fluid samples, the average telomere length of fetuses with a single congenital anomaly was found to be shorter than the average telomere length of control samples, while a significant decrease in telomere length was observed in all anomaly types. Since telomere shortening is more pronounced in cases of multiple congenital anomalies than in fetuses with a single malformation, it is thought that there may be a correlation between telomere length and the severity of the fetal phenotype [10].

Vecoli et al analyzed LTL in 50 adult patients with congenital heart disease exposed to positive medical radiation and 50 healthy controls. They showed that LTL was shorter in the patient group compared to the control group. They stated that LTL shortening in patients may be evidence of premature biological aging [17].

In a study conducted on 236 adolescents born preterm and 38 adolescents born full-term, salivary telomere length was measured. Telomeres were found to be longer in females than in males. The researchers noted gender as a factor significantly associated with telomere length. They also demonstrated a relationship between telomere length and abnormal airflow in a population of adolescents born extremely prematurely [18].

A study in Polish chronic lymphocytic leukemia (CLL) patients examined telomere length and various *hTERT* variants. No significant differences were demonstrated between CLL patients and healthy individuals in terms of MNS16A genotypes and allele frequencies. It has been shown that there is no correlation between telomere length and the age of CLL patients and that there is no relationship between the MNS16A variant and susceptibility to CLL. However, researchers reported that the rs35033501 A allele in the *hTERT* gene is more common in CLL patients and may affect disease susceptibility [16].

Fabio et al. investigated the relationship of the hTERT- MNS16A-VNTR variant with longevity in their study including 1072 healthy individuals from the Central Italian population. The hTERT-MNS16A-VNTR L allele has been shown to shorten life expectancy. They reported that this variant may affect the human lifespan by affecting telomere shortening [19].

In a study conducted with Alzheimer's disease, variants of the *hTERT* gene were examined in 220 individuals diagnosed with late-

onset Alzheimer's and 146 healthy individuals. The hTERT- MNS16A-VNTR LL genotype was found to be associated with an increased risk of Alzheimer's disease only in men. The hTERT-MNS16A- VNTR L allele has been reported to reduce *hTERT* expression and is associated with shorter LTL [15].

Our results, for the first time in these analyses in microtia patients, show that the hTERT-MNS16A-VNTR variant may not be associated with microtia, but telomere shortening may have an association with microtia. Since microtia is a rare congenital anomaly with varying prevalence among populations, studies in different ethnicities and with larger sample groups will further elucidate the relationship between microtia and LTL/hTERT-MNS16A-VNTR.

Since microtia is a rare congenital anomaly, the small sample group used in the study is one of the limitations of the study. The small sample group may not reflect the general characteristics of the Turkish population. Further research with larger populations is needed to confirm the result.

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Patient consent for publication

Informed consent was obtained as written forms from all of the patients to publish.

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