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Cytokine gene variants/expressions and non-syndromic microtia – is there a link?

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Abstract

Objective: Although many genetic and environmental factors are investigated the etiopathogenesis of microtia, it still remains unclear. We investigated the relationship between the variants/expression of pro- and anti-inflammatory cytokines [interleukin (IL) 6, IL-10, tumor necrosis factor-alpha (TNF-α), transforming growth factor beta (TGF-β1), interferon gamma (IFN-γ)] and susceptibility non-syndromic microtia in a Turkish cohort.

Methods: Nineteen unrelated cases with microtia and 40 healthy controls were included in the present study. Cytokine variants were tested by polymerase chain reaction with sequence-specific primers (PCR-SSP) method.

Results: It was found that IL-6 (-174) GG genotype (high expression) was higher in microtia cases than the controls (p=0.010) while IL-6 (-174) GC (high expression) genotype was lower in patients (p=0.003). For IL-6 (-174), patients with GG genotype had a 5895-fold increased risk for microtia. IFN-γ (+874) variant AA genotype (low expression) was lower in microtia cases (p=0.009). IL-6 (-174) G allele was more prevalent in patient group compared to controls while C allele was lower in patients than controls (p=0.003). IFN-γ (+874) variant T allele was more prevalent in cases while A allele was lower in cases (p=0.017).

Conclusion: We have demonstrated for the first time that the cytokine variants constitute risk factors for developing microtia. Our study suggests that the IFN-γ (+874) and IL-6 (-174) variants may be considered as a risk factor for microtia in a Turkish cohorts.

Keywords: Non-syndromic microtia, cytokine, variant, expression.

Özet: Sitokin gen varyantları/ekspresyonları ve non-sendromik mikrotia – Bir ilişki var mıdır?

Amaç: Mikrotianın etyopatogenezinde birçok genetik ve çevresel faktörler araştırılmasına rağmen hala belirsizlik vardır. Bu çalışmada bir Türk kohortunda pro- ve anti-enflamatuar sitokinlerin [interlökin (IL) 6, IL-10, tümör nekroz faktör alfa (TNF-α), transforme edici büyüme faktörü beta (TGF-β1), İnterferon gama (IFN-γ)] varyant/ekspresyonu ve sendromik-olmayan mikrotiaya yatkınlık arasındaki ilişkiyi araştırdık.

Yöntem: Çalışmaya akraba olmayan 19 mikrotiyalı olgu ve 40 sağlıklı gönüllü kontrol dahil edildi. Sitokin varyantları dizi spesifik primer-polimeraz zincir reaksiyonu (PCR-SSP) metodu kullanılarak analiz edildi.

Bulgular: IL-6 (-174) GC genotipi (yüksek ekspresyon) mikrotia vakalarında daha düşükken (p=0.003), IL-6 (-174) GG genotipi (yüksek ekspresyon) mikrotia vakalarında kontrolden daha yüksek olarak bulundu (p=0.010). IL-6 (-174) için, GG genotipi taşıyan hastalar mikrotia için 5895 kat yüksek riske sahipti. IFN-γ (+874) varyant AA genotip (düşük ekspresyon) mikrotia vakalarında düşüktü (p=0.009). IL-6 (-174) C alleli hastalarda kontrollere göre düşükken, G alleli hasta grubunda kontrole göre daha yaygındı (p=0.003). IFN-γ (+874) varyant A alleli hastalarda düşükken, T alleli hastalarda daha yaygındı (p=0.017).

Sonuç: Burada mikrotia gelişimi için sitokin varyantlarının risk faktörü teşkil edeceğini ilk defa gösterdik. Sonuçlarımız IFN-γ (+874) ve IL-6 (-174) varyantlarının Türk toplumunda mikrotia gelişimi ile ilişkili olabileceğini öne sürmektedir.

Anahtar sözcükler: Sendromik olmayan mikrotia, sitokin, varyant, ekspresyon.

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Microtia, manifested by a small, abnormally shaped auricle, is among the most common external ear anomalies; the estimated prevalence varies between 0.8 and 4.2 per 10,000 births depending on the population.[1] It is more common in males, particularly the isolated form. The right ear is more frequently involved.[2] Many factors have been implicated in the etiology, including genetic mutations, vascular abnormalities, and teratogenic agents. Multifactorial inheritance is usually thought to be the most possible cause; however, this is still debatable.[3]

Cytokines are crucial regulators of both maternal receptivity and embryo competence for implantation. There are now numerous studies reporting that cytokines play a role in embryo development, implantation, trophoblast invasion and placental development. Interleukin 6 (IL-6) is a cytokine, secreted by endometrial epithelial cells. It was shown to be related with improved blastocyst development and implantation rates. IL-10 is a pleiotropic cytokine and was shown to regulate resistance to inflammatory stimuli as lipopolysaccaride by down-regulating the amount of pro-inflammatory cytokines in the uterus and placenta.[4] The transforming growth factor beta (TGF-β) superfamily of growth factors contains more than 30 different members. The ligands and their downstream pathway components are very well preserved during evolution, and they regulate various cellular functions. Their actions are regulated during embryonic development, resulting in a variety of astonishing cellular responses.[5] Interferon gamma (IFN-γ) is a pro-inflammatory cytokine produced in the utereus during early gestation. Studies in mice reported that a localized and punctual synthesis of IFN-γ by uterine natural killer cells plays a role in normal placental development and pregnancy outcome.[6] Tumor necrosis factor-alpha (TNF-α) is a cytokine with numerous functions and was identified in the ovary, fallopian tubes, uterus, and placenta, and it is expressed in embryonic tissues almost at all stages of development.[7]

We investigated the relationship between the variants of key pro- and anti-inflammatory cytokines [IL-6 (-174), IL-10 (-1082,-819,-592), IFN-γ (+874), TGF-β1 (codon 10 and 25), TNF-α (-308)] and susceptibility microtia in a Turkish cohort. In addition, expression levels of these cytokines signified in kit procedure were evaluated.

Materials and Methods

Study population

The study group consisted of 19 subjects with microtia, and 40 unrelated healthy control subjects with no personal or family history of dysmorphic disorders. Subjects with microtia were recruited consecutively and prospectively from those who were treated and followed-up in the Plastic, Reconstructive and Aesthetic Surgery Department. All subjects, patients and controls were of Turkish origin. Healthy control group was recruited from the patients living in the same geographical areas, and they were well-matched with the patient group in terms of gender, age and ethnicity. The protocol of this study was approved by the Institutional Ethics Committee, and all subjects gave written informed consent before enrolling in the study.

Genotyping

Whole blood was collected in EDTA tubes and genomic DNA was extracted using salting out method[8] and stored at -20oC until analysis. Cytokine genotyping was performed by the polymerase chain reaction sequence-specific primer method (PCR-SSP), using the Cytokine Genotyping Tray kit according to the manufacturer's instructions. Single nucleotide variants for five cytokines IL-6 (-174), IL-10 (-1082,-819,-592), IFN-γ (+874), TGF-β1 (codon 10 and 25), TNF-α (-308) were analyzed previously described by Karaoglan et al.[9] The expression levels of these cytokines signified in kit procedure were evaluated.

Statistical analysis

Statistical analysis was performed using software SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). The statistical significance of the differences among the groups was estimated by logistic regression analysis. Odds ratio (OR) and 95% confidence interval (CI) were also calculated. Differences in the genotype distribution among the two groups were compared with chi-square test and, when needed, Fisher's exact test was used. The Hardy-Weinberg equilibrium (HWE) was determined using a software analysis.[10] The level of significance was set at $p \leq 0.05$.

Results

Genotype distributions and allele frequencies of TNF-α (-308), IL-6 (-174), and IFN-γ (+874) variants are presented in Table 1. TNF-α (-308) genotype distribution was not significantly different between microtia cases and healthy controls. All cases and controls were in HWE (HWEpa: 0.739, HWEpb: 0.906). A statistically significant difference in the genotype distribution between microtia cases and healthy controls was found for IL-6 (-174) variant. It was noted that IL-6 (-174) GG genotype (high expression) was higher in microtia cases than the

Table 1. Genotype distribution of TNF-α (-308), IL-6 (-174) and IFN-γ (+874) variants between cases and controls.

	Genotype-expression	Microtia cases n^a (%)	Controls n^b (%)	OR*	%95 CI*	p*
TNF-α (-308)	GG (high)	18 (94.7)	36 (89.2)			
	AG (high)	1 (5.3)	4 (10.8)	0.052	0.052–4.807	1.000
IL-6 (-174)	GG (high)	17 (88.2)	20 (50)	5.895	1.482–23.442	**0.010**
	GC (high)	2 (11.8)	17 (43.2)	0.176	0.036–0.870	**0.033**
	CC (low)	0 (0)	3 (6.8)	0.925	0.847–1.010	0.544
IFN-γ (+874)	TT (high)	5 (26.3)	5 (12.2)	2.500	0.625–9.996	0.266
	AT (intermediate)	12 (63.2)	17 (43.2)	2.319	0.754–7.132	0.170
	AA (low)	2 (10.5)	18 (44.6)	0.144	0.029–0.707	**0.009**

TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6, IFN-γ: interferon gamma.
n^a=19, n^b=40, *Fisher's exact test.
The statistically significant results are showed in bold.

controls (p=0.010, OR: 5.85, 95% CI: 1.482–23.442). IL-6 (-174) GC (high expression) genotype was lower in patients, (p=0.003, OR: 0176, 95% CI: 0.036–0.870). The genotype distribution among all cases and the controls was concordant with the HWE (HWEpa: 0.808, HWEpb: 0.813).

There was a significant difference for genotype distribution of (+874) variant of IFN-γ gene between groups. IFN-γ (+874) variant AA genotype (low expression) was lower in microtia cases (p=0.009, OR: 0.144, 95%CI: 0.029–0.707). All groups were in HWE (HWEpa: 0.191, HWEpb: 0.754).

Statistical analysis showed that IL-10 (-1082, -819, -592) and TGF-β (codon 10 and 25) genotypes did not significantly differ between the patient and control groups [data not shown (p>0.05).]. When the IL-10 and TGF-β1 haplotype expressions were compared, no statistically significance was found between the groups. IL-10 deviated from HWE in the groups. The genotype distribution of TGF-β1 among the groups was in line with HWE.

Allele frequencies of TNF-α (-308), IL-6 (-174) and IFN-γ (+874) variants are given in Table 2. It was found that allele frequencies of IL-6 (-174) and IFN-γ (+874) variants showed statistically significant difference between the microtia cases and the controls. IL-6 (-174) G allele was higher in microtia group compared to controls while C allele was lower in microtia groups than controls (p=0.003, OR: 7.263, 95% CI: 1.614–32.680). IFN-γ (+874) variant T allele was more prevalent in cases while A allele was lower in cases (p=0.017, OR: 0.370, 95% CI: 0.168–0.819).

Table 2. Allele distribution of TNF-α (-308), IL-6 (-174) and IFN-γ (+874) variants between cases and controls.

	Alleles	Microtia cases n^a (%)	Controls n^b (%)	OR*	%95 CI*	p*
TNF-α (-308)	G	37 (97.37)	76 (95.00)			
	A	1 (2.63)	4 (5.00)	1.947	0.210–18.042	**1.000**
IL-6 (-174)	G	36 (94.74)	57 (71.25)			
	C	2 (5.26)	23 (28.75)	7.263	1.614–32.680	**0.003**
IFN-γ (+874)	T	22 (57.89)	27 (33.75)			
	A	**16 (42.01)**	**53 (66.25)**	**0.370**	**0.168-0.819**	**0.017**

TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6, IFN-γ: interferon gamma.
n^a=38, n^b=80, *Fisher's exact test.
The statistically significant results are showed in bold.

Discussion

Microtia is a congenital malformation of the ear, ranging from minimal abnormalities to major structural changes or even total absence of the external ear (anotia).[11] Furthermore, most of the cases with microtia also have conductive hearing loss on the affected side.[12] Microtia may be isolated, or manifested as an element of anomalies or a syndrome.[11] Several genetic methods have been used to study microtia, such as linkage analysis, direct sequencing of DNA, investigation of single gene disorders associated with microtia, searching for cytogenetic rearrangements in patients, and the study of animal models. Data for a crucial genetic relationship with microtia revealed higher concordance in monozygotic twins compared to dizygotic twins, 38.5% and 4.5%, respectively.[11,13] Familial cases with autosomal recessive or dominant modes of inheritance with variable expression and incomplete penetrance were also reported.[14] Prevalence of familial cases was estimated between 3 and 34%.[11,15] Mouse models showed that mutations in specific genes could result in microtia.

Immunologic system has a crucial function that enables normal pregnancy development and promotes the development of complications. Success in pregnancy outcome seems to be closely related to a distinct balance between the cytokines Th1 and Th2, both involved in fetal growth and development. In this study, we investigated the relation between the IL-6 (-174), IL-10 (-1082,-819,-592), IFN-γ (+874), TGF-β1 (codon 10 and 25), and TNF-α (-308) variants and susceptibility to microtia in a Turkish cohort. Furthermore, we evaluated expression levels of these cytokines signified in kit procedure.

IL-6 is a marker of inflammation and it plays a pro-inflammatory and anti-inflammatory mediator role. IL-6 is expressed in a various cell types, including blood cells, fibroblasts, macrophages and adipose cells. IL-6 was also shown to be expressed in the fallopian tubes of humans and pigs. IL-6 synthesized in endometrial epithelial cells is associated with improved blastocyst development and implantation rates.[16] IL-6 was also reported to enhance cell number and decrease apoptosis in mouse blastocyts.[17] IL-6 knockout mice are fertile; however, their fertility is diminished, implantation rates are low and miscarriage rates in mid-gestation are high. The human IL-6 gene has about 50 variants in its promoter region.[18] A functional -174GC (rs1800795) variant is related to the fundamentally IL-6 transcription rate, which could affect the level of serum IL-6. Compared to -174CC genotype carriers, -174 GG/GC carriers have a higher IL-6 expression.[17] In the present study, we found that IL-6 (-174) variant genotype distribution was related with microtia (Table 1). IL-6 (-174) GG genotype (high expression) was higher in microtia cases than the controls (p=0.010) while IL-6 (-174) GC (high expression) genotype was lower in patients, (p=0.003). It seems that GC genotypes create protection advantage of heterozygosity for microtia. Also, IL-6 (-174) G allele was more prevalent in microtia group compared to control group while C allele was lower in microtia cases than controls (p=0.003) (Table 2).

IFN-γ is crucial in several cellular processes, such as inducing innate and adaptive immune responses, hindering cell proliferation and stimulating apoptosis.[19] It is mainly secreted by CD4 +Th cells, CD8+T cytotoxic cells, and natural killer cells. Research in mice suggested that a localized and punctual production of IFN-γ by uterine natural killer cells facilitates normal placental development and pregnancy outcome.[6] The human IFN-γ gene has a variant in the first intron at its 5' end, next to a CA repeat region (+ 874 A>T, rs2430561) that affects the synthesis of IFN-γ and had been associated with several autoimmune and chronic inflammatory conditions.[20] Investigation of the biological role of this variant showed that + 874 T allele carriers had higher production of IFN-γ.[20] We found that IFN-γ (+874) variant AA genotype (low expression) was lower in cases (p=0.009) (Table 1). It was thought that IFN-γ AA genotype had protective role against microtia. Also, IFN-γ A allele was lower in microtia cases (p=0.017) (Table 2).

TNF-α is a cytotoxic protein which arises after endotoxin treatment in rabbit serum. TNF-α acts as a mediator in apoptosis processes and is involved in infection and immune reactions. It plays a significant role in the etiopathogenesis of several diseases: sepsis, multiple sclerosis, osteoporosis, some types of cancer and diabetes.[21] Studies conducted in the past few years have noted the importance of TNF-α in reproductive medicine. It seems to have an impact on pregnancy. Therefore, TNF-α protein, TNF-α RNA and also its receptors TNF-R1 and TNF-R2 are expressed during the pregnancy in various tissues including ovaries, endometrium, placenta and in the fetus itself.[21] It has been known that TNF-α wields deleterious effects on pre-implantation embryo development. TNF-α given to mice during early pregnancy had a detrimental effect on implantation or reduced litter size in mice and rats.[22] TNF-α has been held responsible in embryopathies occurring due to developmental toxicants, various stresses, and maternal metabolic derangements. TNF-α appears to involve in the differentiation and growth processes in a normal pregnancy. In the present

study, TNF-α genotype distribution showed no association between microtia cases and controls (p>0.05). Also, we found no significant difference according to haplotype analysis between groups (Tables 1 and 2).

In humans, IL-10 is encoded by five exons and four introns and is located at the 1q31-32 position. IL-10, a pleiotropic cytokine, affects in the process of inflammation and immunoregulation. It is likely that IL-10 is a major cytokine for the maintenance of pregnancy thanks to its protective effect on the feto-placental unit. It hinders the synthesis of inflammatory cytokines including IL-6, TNF-α and IFN-γ. Along with IL-4 and IL-13, IL-10 seems to regulate trophoblast invasion and to facilitate placental development.[23] Recent studies reported that some biologically important variants were present in the IL-10 gene. These variants may affect the interleukin-10 production rate. Synthesis of IL-10 is modulated at transcriptional, posttranscriptional and translational levels.[24] These variants are found at positions -592 (rs1800872, A/C), -819 (rs1800871, T/C) and -1082 (rs1800896, A/G) (24). In this study, we noted no association between IL-10 genotype distribution/haplotype analysis and microtia (p>0.05).

TGF-β superfamily is a group of signaling factors such as TGF-β and bone morphogenetic proteins (BMPs) with a significant capability to stimulate cartilage and bone.[25] In the embryonic phase, chondrocytes develop in distinct phases of cell proliferation, condensation and maturation to proliferating chondroblasts synthesizing collagen type II and proteoglycans. Exogenous TGF-β induces embryonic development in vitro, facilitating blastocyst proliferation and development and raising the number of blastocysts.[26] Several TGF-β superfamily members, their receptors and Smads are expressed in embryos during late phases. They also found to be involved in gastrulation and organogenesis.[26] In this study, we identified no statistically significant association between the cases and the control subjects according to genotype distribution and haplotype analysis of the TGF-β1 (codon 10 and 25).

Conclusion

While the precise pathogenesis of microtia remains ill-defined, it is believed to be a multifactorial malformation in which environmental and genetic factors are involved. As far as we know, the association between cytokine variants and microtia has not been investigated previously in a Turkish population. Our data suggest that the IL-6 (-174) and IFN-γ (+874) variants may have a functional effect in microtia. Our data provide a genetic basis for the hypothesis that cytokine variants may play a role in the formation of microtia. Larger studies in different ethnic populations are needed to confirm our findings.

Conflict of Interest: No conflicts declared.

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