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Association of *UCP2* G (-866) A Polymorphism, but not *NAT2* G (590) A, with Presbycusis Susceptibility in an Iranian Population

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Abstract

Background: Presbycusis, or age-related hearing loss, is a common sensory disorder in older adults. Oxidative stress is a major pathogenic mechanism, and polymorphisms in genes regulating detoxification and reactive oxygen species (ROS) balance, such as *NAT2* and *UCP2*, may influence disease susceptibility.

Methods: We conducted a case–control study including 120 male presbycusis patients (mean age 65.1 years) and 120 age-matched healthy controls (mean age 63.4 years). Genotyping of the *NAT2* 590G>A (rs1799930) and *UCP2* G (-866) A (rs659366) polymorphisms was performed using PCR-RFLP. Statistical analyses included odds ratios (ORs) with 95% confidence intervals (CIs), logistic regression, and Hardy–Weinberg equilibrium testing. Bioinformatics tools (including the UCSC Genome Browser, ORegAnno, and CpG island analysis) were used to predict the functional effects of variants.

Results: No significant association was observed between *NAT2* 590G>A and presbycusis risk (OR = 1.22, 95% CI = 0.84–1.79, p = 0.289). In contrast, the UCP2 G(-866)A polymorphism showed a strong association: the AA genotype (OR = 3.20, 95% CI = 1.22–8.41, p = 0.018), A allele (OR = 1.68, 95% CI = 1.14–2.47, p = 0.008), and dominant GA+AA vs. GG model (OR = 1.84, 95% CI = 1.10–3.08, p = 0.02). Bioinformatics analysis indicated that the polymorphism may alter CpG islands in the promoter region and may could affect transcription factor binding and gene expression.

Conclusion: Our findings suggest that the *UCP2* G (-866) A polymorphism, but not *NAT2* 590G>A polymorphism, contributes to presbycusis susceptibility in the studied Iranian population. This variant could serve as a potential biomarker for the genetic risk assessment of presbycusis.

Keywords: Age-Related, Arylamine N-Acetyltransferase, Genetic, Hearing Loss, Polymorphism, Presbycusis, Uncoupling Protein 2.

Introduction

Presbycusis or age-related hearing loss (ARHL) is one of the most common age-related diseases. Hearing dysfunction caused by this disease is progressive, irreversible, and, symmetrical, bilateral and occures in the cochlea of the inner ear. People first experience hearing impairment at high frequencies, then they perceive sounds more poorly, and then have problems understanding words. This disease is characterized by mild (low risk), moderate, and severe (high- risk) stages (1-3).

Presbycusis can start at an early age, but it usually occurs in individuals aged 60 years and older. According to reports from the World Health Organization, about 1.2 billion people are expected to be affected by 2025(2, 4). To date, no definitive treatment has been discovered for patients with presbycusis, but there are some ways to prevent or control it at a younger age. However, researchers are trying to find new methods for the definitive

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treatment of presbycusis, including gene therapy and stem cell therapy (2, 5).

Presbycusis is a multifactorial disorder in which both genetic and non-genetic (environmental) factors are involved. Environmental factors are divided into two categories: external factors, such as lifestyle (including alcohol consumption and cigarette smoking), noise, and chemical exposure (including industrial chemical toxins) and individual health factors, such as age, ototoxic medications (aminoglycoside antibiotics, salicylates), and chronic diseases (including diabetes, obesity, and cardiovascular disease) which underlie this disorder (2, 6). Important mechanisms involved in the pathogenesis of presbycusis include inflammation, apoptosis, mitochondrial dysfunction, and oxidative stress.

The oxidative stress pathway plays a crucial role in the pathogenesis of presbycusis disease. Oxidative stress is a process caused by the excessive accumulation of reactive oxygen species (ROS) in many tissues and damage to cells (7, 8). Produced ROS by mitochondria cause damage to mitochondrial components and eventually lead to disruption of mitochondrial function (9). The body has developed an antioxidant system against ROS to counteract ROS and prevent cellular disfunction. In this pathway, antioxidant enzymes, including N-acetyltransferase (*NAT*) (10), cytochrome P450 (*CYP1A1*), glutathione S-transferase (*GST*) such as *GSTT1, GSTM1*, *GSTP1* (11), and uncoupling proteins (UCPs) are involved (10).

NAT2 is an enzyme that is responsible for the elimination and detoxification of exogenous substances by N-acetylation and O-acetylation. Genetic changes in the *NAT2* gene lead to alterations in the rate of isoniazid acetylation. The activity of the acetyltransferase enzyme is reduced in carriers of the mutant alleles, which are called slow acetylators. The G590 >A polymorphism encodes a slow acetylator that impairs the detoxification mechanism and ultimately leads to the accumulation of xenobiotics in the inner ear. The association between NAT2 590G>A and presbycusis was first reported by Ünal et al in the Turkish population at first (12). Subsequently, this association was also confirmed in other populations (10, 13).

UCP2 is an important transporter protein that is also involved in the oxidative stress pathway, which that facilitates the transport of anions and controls the production of ROS in mitochondria. Genetic variations in this gene lead to an increase in ROS and cell damage in the inner ear. Recent studies have confirmed the association between G-866A polymorphism of the *UCP2* gene and obesity (14) and diabetes (15) but there is only one study about this single nucleotide polymorphism (SNP) and presbycusis disease in the Indian population (10).

In this study, we aim to investigate the association between 590G>A, single nucleotide polymorphisms of the *NAT2* gene and G-866A of the *UCP2* gene, which both are important genes in the oxidative stress pathway, and the risk of presbycusis disease in an Iranian population.

Materials and Methods

Subjects

Presbycusis (age-related hearing loss) was defined as a bilateral, symmetrical, progressive sensorineural hearing loss in adults aged ≥60 years of age, consistent with the World Health Organization (WHO, 2021) grading of hearing impairment. According to WHO, adult hearing loss is considered clinically significant when the pure-tone average (PTA) at 0.5, 1, 2, and 4 kHz in the better ear exceeds 20 dB HL, with severity classified as mild (21–35 dB), moderate (36–50 dB), severe (66–80 dB), or profound (≥81 dB) (16, 17).

A total of 120 male healthy controls (average age: 63.4 ± 5.2 years) and 120 male presbycusis patients (average age: 65.1 ± 5.0 years) were selected from Matini Hospital in Kashan (Isfahan, Iran) during 2016-2020. All participants were from the same geographic region and ethnic origin (Iranian-Caucasian). All presbycusis patients were newly diagnosed at first presentation and, in accordance with the above criteria, had the following characteristics:

were over 60 years old, had a healthy eardrum, and had not undergone any ear-related surgery, had no history of trauma to the temporal bone, and had hearing impairment above 30 dB. Likewise, the control subjects did not have any hearing disorders or ear-related surgery. Both control and patient groups were selected from people with no history of noise exposure, chronic history including chronic otitis media, hearing impairment caused by ototoxic drugs, diabetes, cardiovascular diseases, chronic degenerative neurological diseases, cancer, obstructive lung disease, allergy, and hepatitis. Cases and controls were age- and gender-matched, as both groups consisted of male participants with no significant difference in mean age ($p > 0.05$).

DNA isolation

Two milliliters of blood were collected from all participants in tubes containing EDTA (Merck, Germany) and stored at -20 °C. Genomic DNA was extracted using using the Bioneer kit (Tehran-Iran) and stored at -80 °C for future use.

NAT2 and *UCP2* Genotyping

The polymorphism 590G>A of the *NAT2* gene and G-866A of the *UCP2* gene were genotyped using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP). Primer sequences, PCR programs, and amplification product sizes are summarized in Table 1. Primers were synthesized by Metabion (Germany). PCR was performed in a reaction volume of 25 µl containing: 12.5 µl Master Mix (2X PCRBIO Taq Mix), 6.5 µl H_2O, 1 µl of each of reverse and forward primers, and 4 µl of a DNA sample. The PCR program was identical for both SNPs and consisted of an initial denaturation at 94 °C for 8 minutes, followed by 35 cycles of 94 °C for 45 seconds, annealing at the specific primer temperature for 45 second, and extension at 72 °C for 45 seconds, with a final extension at 72 °C for 8 minutes. The annealing temperature was 60 °C for *NAT2* and 64 °C for *UCP2***.**

Table 1. Primer sequences and amplicon sizes.

Gene	SNP, (rs No.)	Primer sequence	Product size (bp)
NAT2	590 G>A, (rs1799930)	(F): 5'-ACGGCAGGAATTACATTGTC-3'	361
		(R): 5' ACCAAACAGTAAACCCCTTC -3'	
UCP2	866 G>A, (rs659366)	(F): 5'- TTCTGTTTCCACGCTGCTTC -3'	472
		(R): 5'- TCACGCTCCTACACACAGAC -3'	

F: Forward, R: Reverse.

The PCR products were digested with *Taq*I (Thermo Scientific, Lithuania) at 65 °C for 16 hours and *Mlu*I (Thermo Scientific, Lithuania) at 37 °C for 16 hours. Fragments were separated and visualized on 2% agarose gels.

Bioinformatics analysis

The UCSC Genome Browser (http://genome.ucsc.edu) was applied to study bioinformatically and visualize the regulatory region around G-(866)A polymorphism in *UCP2* gene. We utilized Open Regulatory Annotation (ORegAnno database) and CpG Islands databases for regulatory analysis. CpG islands are short, dispersed regions of unmethylated DNA with a high frequency of CpG dinucleotides relative to the bulk genome (18). They are typically common near transcription start sites and may be associated with promoter regions and with the regulation of gene expression. In this research, CpG Islands in the GC-rich promoter region of *UCP2* were examined. The ORegAnno database describes experimentally validated and published regulatory regions (promoter,

enhancer, etc), transcription factor binding site, or regulatory polymorphism (19, 20).

Statistical analysis

The genotypes were tested for Hardy-Weinberg equilibrium (HWE) in both patient and control groups using the Chi-square test. The association of each allele and genotype with presbycusis risk was evaluated with an odds ratio (ORs) and 95% confidence intervals (CI).

ORs and 95% CI were calculated using logistic regression test. A p-value of <0.05 was considered statistically significant. Statistical analysis was done by SPSS software version 19.

Results

Genotyping results

The PCR products of the *NAT2*:590G>A and *UCP2*:G(-866)A polymorphisms after digestion by the *Taq*I and *Mlu*I enzymes, respectively are shown in Figure 1. The direct DNA sequencing revealed that PCR-RFLP results for the three mentioned SNPs were reliable (Fig.2). The digested products are summarized in Table 2.

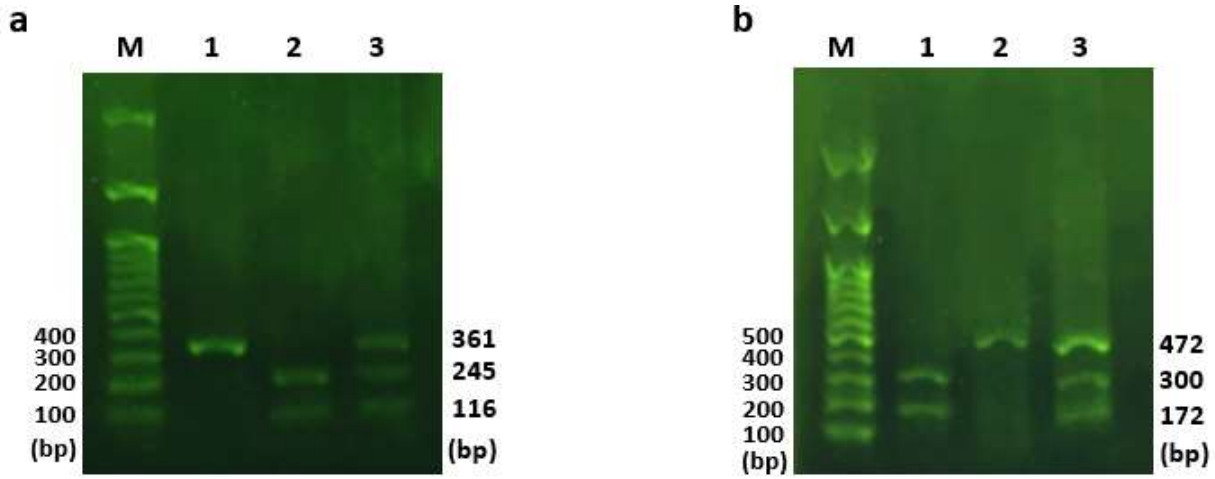

Fig. 1. a) Restriction fragment length polymorphism analysis of *NAT2* 590G>A in agarose gel. PCR products were digested with *Taq*I enzyme. Samples 1, 2, and 3 are defined with AA, GG, and AG genotypes, respectively. **b)** Restriction fragment length polymorphism analysis of *UCP2* G (-866) A in agarose gel. PCR products digested with the *Mlu*I enzyme**.** Samples 1, 2, and 3 are defined with GG, AA, and AG respectively. M: 100bp DNA marker.

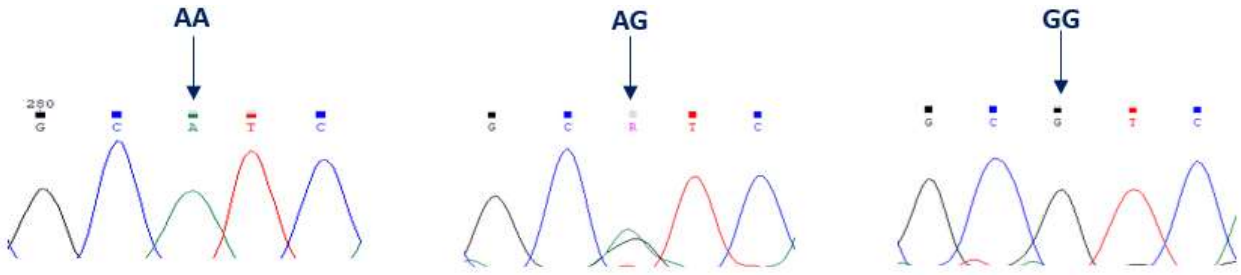

Fig. 2. Analysis of direct-sequence result of *UCP2* G (-866) A polymorphisms. DNA sequencing of the representative samples shows three genotypes (AA, AG, and GG) in *UCP2* gene for G (-866) A polymorphism.

Table 2. Digestion conditions of the restriction endonucleases.

Gene	SNP (rs No.)	Restriction enzyme	Restriction site	Digestion product size (bps)
NAT2	(rs1799930)	*Taq*I	5′-TCGA -3′	GG= 245,116 GA= 361,245,116 AA= 361
UCP2	(rs659366)	*Mlu*I	5′-ACGCGT -3′	GG= 300, 172 GA= 472, 300, 172 AA= 472

Distribution of genotypic and allelic frequency of NAT2 590G>A and UCP2 G (-866) A SNPs

The results of the genetic association study for 590G>A polymorphism of the *NAT2* gene and G (-866) A polymorphism of the *UCP2* gene are summarized in Table 3. Both studied polymorphisms were in Hardy- Weinberg equilibrium in case and control groups.

According to the results of statistical analysis, there was no significant association between rs1799930 and presbycusis (Table 3). No difference in the frequency of the A allele was observed between the case and control groups (OR = 1.22, 95% CI = 0.84-1.791, p = 0.289).

Table 3. Association analysis of rs1799930 (G590A) and rs659366 (G-866A) with presbycusis risk.

SNPs	N (%)		OR (95%cI)	*P*-value	HWE χ2 (p value)	
	Controls (n=120)	Cases (n=120)			Controls	Cases
rs1799930 G>A						
GG	59 (49.1%)	51 (42.5%)	-	-	0.21	0.378
GA	46 (38.3%)	51 (42.5%)	1.282 (0.742-2.216)	0.372		
AA	15 (12.5%)	18 (15%)	1.388 (0.635-3.031)	0.410		
(AA+AG)/GG	61 (50.8%)	69 (57.5%)	1.308 (0.786-2.177)	0.3		
AA/(AG+GG)	15 (12.5%)	18 (15%)	1.235 (0.590 - 2.582)	0.57		
GA/(GG+AA)	46 (38.3%)	51 (42.5%)	1.189 (0.709 - 1.992)	0.51		
Alleles						
G	164	153	-	-		
A	76	87	1.22 (0.84 - 1.791)	0.289		
rs659366 G>A						
GG	63 (52.5%)	45 (37.5%)	-	-	0.474	0.621
GA	50 (41.6%)	59 (49.1%)	1.652 (0.965 - 2.826)	0.066		
AA	7 (5%)	16 (13%)	3.200 (1.216 - 8.416)	**0.018***		
(AA+AG)/GG	57 (47.5%)	75 (62.5%)	1.842 (1.101 - 3.082)	**0.02***		
AA/(AG+GG)	7 (5%)	16 (13%)	2.483 (0.982 - 6.277)	0.05		
GA/(GG+AA)	50 (41.6%)	59 (49.1%)	1.354 (0.813 - 2.254)	0.24		
Alleles						
G	176	149	-	-		
A	64	91	1.679 (1.14 - 2.473)	**0.008****		

* $p < 0.05$, ** $p < 0.01$, N: number.

Regarding G-866A polymorphism of the *UCP2* gene, the frequency of the AA genotype was significantly increased in the presbycusis patients when compared to the controls, and this is significantly associated with the risk of presbycusis (OR = 3.20, 95% CI = 1.216-8.416, p = 0.018). Likewise, there was a significant association between the A allele and presbycusis susceptibility (OR = 1.679, 95% CI = 1.14-2.473, p = 0.008). We further found an increased risk of presbycusis in patients in GA + AA vs. GG dominant model compared to healthy people (OR = 1.84, 95% CI = 1.10-3.08, p = 0.02). However, there was no significant association with presbycusis in GG + GA vs. AA recessive model (OR = 2.4835, 95% CI = 0.9826-6.2774, p = 0.05), or in GG + AA vs. GA co-dominant model OR = 1.3541, 95% CI = 0.8134-2.2543, p = 0.24).

Discussion

Aging is a genetic process that progresses over time, leading to degeneration of body organs, and is driven by biological changes. One of the most common age-related disorders which is discussed today, is presbycusis (2). Numerous risk factors contribute to the pathogenesis of this disorder. One of the main pathogenic mechanisms is oxidative stress, a process caused by an imbalance between the production of reactive oxygen species (ROS) and their elimination (21). *NAT2* and *UCP2* genes play an important role in the oxidative stress pathway.

NAT2, an isoenzyme of the NAT enzyme, is highly polymorphic and catalyzes amine and hydrazine aromatic substances, contributing to the balance the oxidative state. 590G>A polymorphism, which is located in exon 2 of the *NAT2* gene, is considered as a slow acetylator phenotype (22). All reported association studies between rs1799930 genetic variation and presbycusis has been summarized in Table 4. The differences in reported studies may be related to population genetic background and ethnicity.

No association between 590G>A polymorphism and presbycusis was not found in this study. However, due to the relatively small sample sizes and population genetic background, research results may be contradictory, and further confirmation is needed through multi-center, large sample population studies.

UCP2 is one of five members of a family of anion transporter proteins located in the inner membrane of mitochondria. This gene is involved in the oxidative stress pathway and regulates the level of ROS. G(-866)A polymorphism of the *UCP2* gene is located in the promoter region that probably may affect gene expression (10, 23). Some studies have confirmed the relationship between this polymorphism and type 2 diabetes (15) and obesity (14) but there is only one study on the association between rs659366 and presbycusis in an Indian population (10).

In the present study, we found a significant correlation between *UCP2* G (-866) A polymorphism and presbycusis disease. This polymorphism is located in the promoter region of the *UCP2* gene. According to the Genome Browser database, there are 96 CpG island regions harboring the G (-866) A polymorphic site where the conversion of G to A alters one of the CpG islands and may consequently affect *UCP2* gene expression.
Additionally, bioinformatics analysis from the Genome Browser (OReg Anno database) demonstrated that transcription factors RBL2, SMARCA4, and PRDM14 bind to this regulatory region of the *UCP2* gene (Table 5). In reviewing the literature, we found an association between RBL2 and SMARCA4 and hearing dysfunction. The *RBL2* gene is one of the retinoblastomas (pRB) family of cell cycle regulators, which regulate the G1- to S-phase transition in proliferating cells. Sanchez et al analyzed the cochlea of adult Rbl2/p130 knock-out mice and showed that absence of the *Rbl2* gene results in extra rows of hair cells (HCs) and supporting cells (SCs) in the more apical regions of the cochlea (24). As a result, the loss of Rbl2 affects the development of supporting and hair cells in the inner ear. However, the association between *RBL2* and

human hearing loss has not been reported to date. Overall, the phenotypic features of Rbl2 knock-out mice support a potential link between G (-866) A and presbycusis in human. Although an association between Rbl2 and human hearing loss has not been observed, mutations in mice are known to cause hearing loss or cochlear developmental defects.

Table 4. Summarized association studies between *NAT2* 590G>A(rs1799930) and presbycusis.

Reference	Population	No of Case/control	Statistical significance
(12)	Turkish	68/98	Significant GA (p-value= 0.032) AA (p-value= 0.013)
(13)	American	55/79	Significant GA (p-value= 0.004) AA (p-value= 0.01)
(10)	Indian	220/270	Significant GA (p-value = 0.041) AA (p-value = 0.008)
(26)	Caucasian	145/120	Not significant
Current study	Iran (Kashan)	120/120	Not significant

Table 5. Transcription factors binding to G(-866)A polymorphic region of *UCP2* gene.

ORegAnno ID	Transcription factor	Chromosomal location: transcription factor binding site	Correlation with hearing impairment	Reference
OREG1251703	SMARCA4	Chr11:73979907-73985706	Yes	(25)
OREG1832965	PRDM14	Chr11:73983277-73983777	No	-
OREG1792033	RBL2	Chr11:73982919-73983853	Yes	(24)
OREG1813194	RBL2	Chr11:73982929-73983774	Yes	(24)

SMARCA4, a member of the family of proteins with helicase and ATPase activities, has also reported to be associated with hearing in males (25). This finding supports the importance of G (-866) A polymorphism in presbycusis susceptibility. Regarding PRDM14, no reports were found its association with hearing dysfunction.

Altogether, this mutation in the promoter region of the *UCP2* gene may affect gene expression through alterations in the methylation pattern as well as nucleotide substitution in transcription afctor binding sites. The novelty of our work lies in being the first report from an Iranian population demonstrating the contribution of *UCP2* polymorphism to presbycusis risk. This expands the genetic landscape of presbycusis and highlights the importance of mitochondrial genes in age-related hearing loss research.

In conclusion our findings indicate that G (-866) A polymorphism of the *UCP2* gene could be considered a genetic risk factor for presbycusis susceptibility and may serve as a potential biomarker. However, no significant association was found between *NAT2* 590G>A

polymorphism and presbycusis. Further studies with larger sample sizes are required to evaluate this genetic variation as a potential biomarker for presbycusis risk.

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Conflict of interest
The authors declare that they have no conflict of interest.

Ethics
The study protocol was approved by the ethics committeeof the University of Mazandaran, Babolsar, Iran (IR.UMZ.REC.1400.030). Informed written consent was obtained from all subjects at the beginning of the study.

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Authors' contribution
FK wrote the manuscript, performed the experiments, analyzed the data; OJ supervised the research, participate in manuscript writing, analysis and interpretation the results; MK collected the samples, participate in data analysis and commented the manuscript; EJ commented the manuscript. All authors read and approved the final manuscript.

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