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Research paper

Association of oxidative stress gene polymorphisms with presbycusis

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ABSTRACT

Introduction: Presbycusis is characterised by etiopathological changes in the cochlea of the inner ear due to genetic and environmental factors and has a serious impact on quality of life. The present study was aimed to evaluate the role of oxidant stress gene polymorphisms in the development of presbycusis.
Subjects and methods: 220 subjects with confirmed presbycusis from ENT specialists of MAA ENT hospital, Hyderabad, India from 2012 to 2014 were considered for the study. 270 age and sex matched controls were included in the study. Analysis of gene polymorphisms of SNPs cytochrome P450 1A1 (*CYP1A1*) 3801 T>C, 2455 A>G and 2453 A>C; glutathione S transferase (*GST*) T1 and M1; N-acetyl transferase (*NAT2*) 282 C>T and 857 G>A; uncoupled proteins (*UCP1*) (−3826) A>G and (*UCP2*) (866)G>A was carried out. Variations in the allelic and genotypic frequencies obtained were computed and analysed using appropriate statistical methods.
Results: The results of the study indicated that *CYP1A1* gene polymorphism at 2453 C>A (adjusted OR: 1.59, 95% CI: 1.01–2.87) and 2455 A>G (adjusted OR: 1.87, 95% CI: 1.07–3.37), double null genotype of *GSTM1* and *GSTT1* (adjusted OR: 8.88, 95% CI: 4.10–19.19), *NAT2* gene at C282T (adjusted OR: 1.77, 95% CI: 1.02–3.11) and G590 A (adjusted OR: 1.83, 95% CI 1.20–3.63) and *UCP2* (−866) G>A (adjusted OR: 12.39; 95% CI: 6.51–23.56) showed increased risk for presbycusis while *CYP1A1* at 3801 T>C and *UCP1* (−3286) A>G exhibited no association. The haplotype combinations of **T**-G-A of *CYP1A1* at 3801, 2455 and 2453 positions as well as T-A of *NAT2*6* at 282 and 590 positions were found to contribute significant risk for the onset of presbycusis.
Conclusions: Gene polymorphisms of *CYP1A1 (A2455G, C2453A)*, *NAT2*6* (C282T, G590 A), *GST T1/M1* (double null genotype) and *UCP2* (G-866 A) were found to contribute significant risk to presbycusis.

1. Introduction

Presbycusis is a complex auditory disorder caused by degenerative changes due to aging in the cochlea of inner ear and central auditory pathways that lead to high-frequency hearing loss and loss of speech discrimination (Cruickshanks et al., 1998; Staecker et al., 2001). The cumulative effect of intrinsic and extrinsic factors like anatomical degeneration, noise exposure, medical disorders and their treatment, as well as hereditary susceptibility are reported to affect cochlea function (Bared et al., 2010). Especially, reductions in oxygen and an increase in the production of free radicals (Reactive oxygen species: ROS) leads to increase in the risk for age related hearing loss (Seidman et al., 2002; Coling et al., 2003; Ohlemiller, 2009). Gene polymorphisms of several detoxification and antioxidant phase I and II enzymes such as cytochrome P450 (*CYP1A1*), glutathione peroxidase (*GSTP1*), glutathione S-transferases (*GST* M1 and T1), N-acetyltransferase (*NATs*) increase ROS levels and thereby induce cellular injury (Staecker et al., 2001; Seidman et al., 2002; Hein, 2002). In addition, mitochondrial mutations (*UCP1, UCP2*) acquired due to aging may also contribute to the degeneration process in the cochlea of inner ear and thereby contribute to hearing loss (Gates et al., 1999).

CYP1A1 gene located on chromosome 15q22–24 spans 5810 bp has 7 exons and 6 introns codes for an enzyme aryl hydrocarbon hydroxylase responsible for the metabolism of polycyclic aromatic hydrocarbons (Hayashi et al., 1991). Studies have reported that substitution at position 3801 involves the transition of thymidine to cytosine in the 3′ non-coding region. The other well-known polymorphism *CYP1A1* 2455 A to G is downstream of exon 7 region involves replacement of isoleucine with valine in the catalytic region of the protein that leads to an increase in enzyme activity while 2453 substitution of C to A in exon 7 leads to changes in threonine to aspartate at codon 461 (Hayashi et al., 1991; Landi et al., 1994). Many phase II drug metabolizing enzymes such as the *N*-acetyltransferases (*NAT1* and *NAT2*) and the glutathione *S*-transferases (*GSTM* and *GSTT*) were found to play an important role in the antioxidant protection of adult cochlea (Parl, 2005; Liu and Yan,

Abbreviations: ROS, Reactive oxygen species; CYP, cytochrome P450; GST, glutathione S-transferases; NAT, N-acetyltransferase; UCP, Uncoupled proteins; Ala, Alanine; Val, Valine; Thr, Threonine; PCR, Polymerase chain reaction; RFLP, Restriction Fragment Length Polymorphism.

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2007). The genetic polymorphisms of *GSTM1* and *GSTT1*, involves deletion of 20 base pairs respectively which leads to loss in functional activity towards conjugation specific metabolites and enzymes (Duell et al., 2002). The genes encoding cytosolic N-acetyltransferase consist of an introns less protein coding exon with an open reading frame of 870 base pairs are located at 8p22 (Grant et al., 1989). Genetic variations in N-acetyltransferase genes leads to altered functional activity level of isoniazid acetylation, that has been classified into rapid, intermediate, and slow acetylator phenotypes (Butcher et al., 2002; Sabbagh et al., 2013). Eight different point mutations in *NAT2*, of which five cause amino acid changes at 191 G to A (Arg to Glu);341 T to C (Ile to Thr); 590 G to A (Arg to Gln); 803 A to G (Lys to Arg); 857 G o A (Gly to Glu), and three of them leading to silent amino acid changes at 111 T to C, 282 C to T and 481 C to T were commonly reported (Butcher et al., 2002). Several allelic variants of *NAT2* result from certain combinations of the eight point mutations and the nomenclature of the alleles is based on the classification system of Vatsis et al. (1995). The slow acetylator phenotype for *NAT2* cannot conjugate metabolites or toxins specific to these enzymes and can lead to an increase in the susceptibility to environmental toxins and oxidative free radical cellular damage.

Uncoupling proteins (*UCPs*) are the important carrier proteins which facilitate the transfer of anions, thereby control the mitochondria-derived reactive oxygen species (ROS) (Giardina et al., 2008). *UCP* consists of 1, 2, 3, 4, and 5 members of an anion-carrier protein family which are located in the inner mitochondrial membrane (Souza et al., 2011). The *UCP1* covers a 9 kb region on chromosome 4 (region 4q28-q31), and consists of 6 exons and 5 introns which is mainly expressed in brown adipose tissue (Yu et al., 2005). As *UCP1* has been found to be involved in energy expenditure, several polymorphism especially at −3826 A to G, −1766 A to G, and −112 A to C positions in the promoter region; Ala64Thr polymorphism in exon 2; and Met299Leu polymorphism in exon 5 were found to be associated with pathogenesis (Brondani et al., 2012). *UCP2* is widely distributed and the gene consisting of eight exons and seven introns covers a 6.3 kb region on chromosome 11q13. Polymorphisms contribute to biological variation in insulin secretion but the main function of *UCP2* is the control of mitochondria-derived ROS (Arsenijevic et al., 2000; Krempler et al., 2002). In Japanese population, it was recently reported that *UCP2* gene polymorphism at Ala55Val in exon 4 is significantly associated with age related hearing loss but not with *UCP1* A (−3826) G polymorphism (Sugiura et al., 2010).

Therefore, the damage caused by oxidative stress to auditory system might be causing susceptibility to presbycusis. Hence, the present study is the first of its kind in Indian population to evaluate the role of genes involved in oxidative stress and mitochondrial dysfunction leading to the etiopathogenesis of presbycusis.

2. Subjects and methods

2.1. Subjects

In the present case–control study, 220 presbycusis cases referred to MAA ENT HOSPITALS, Hyderabad, Telangana State, from 2011 to 2014 and 270 age and sex matched controls were included as study subjects. All patients underwent a detailed medical otoscopic examination that includes tympanometry and pure tone audiometric test for evaluating hearing loss at 0.5, 1, 2, 4 and 8 kHz frequencies. The patients with sensorineural form of hearing loss (bilateral as well as symmetrical) whose age was equal or greater than 40 years and with no vestibular or any history of otological surgery were included in the study. Outer and middle ear diseases that include infections as well as patients residing/working in noisy environment were excluded from the study. Individuals without any disease were considered as healthy controls. Informed written consent was taken from all participants, and the study was carried out with the Institutional Ethics committee approval. Blood samples were collected from all study subjects and were kept in EDTA vials for analysis. Genomic DNA was isolated from whole blood samples by salting out method of Lahiri and Nurnberger (1991) and stored in −80 °C for molecular analysis.

2.2. CYP1A1 genotyping

The *CYP1A1* 3081 T>C polymorphism was detected by PCR based Restriction Fragment Length Polymorphism (PCR-RFLP) (Cascorbi et al., 1996). The PCR was carried out with specific forward primer (5′-GGCTGAGCAATCTGACCCTA-3′) and reverse primer (5′-TAGGAGTCTTGTCTCATGCCT-3′). After mixing all the contents, PCR tubes were kept in thermal cycler for a 3 step PCR with an initial denaturation at 95 °C for 5 min followed by cycling at 95 °C for 30 s, annealing for 60 °C at 60 s and extension at 72 °C for 60 s and a final extension at 72 °C for 5 min was carried out for about 30 cycles. The PCR product 899 bp was digested with *MSPI* restriction enzyme at 37^0 C for 5 min and electrophoresis was carried out on 1.5% agarose gel with ethidium bromide staining. The RFLP products showed homozygous 'TT' genotype with 899 bp, heterozygous 'TC' genotype with 899, 693, 206 bp and homozygous 'CC' genotype with 693, 206 bp fragments. *CYP1A1* was checked by amplifying a 204 bp fragment using forward (5′-CTGTCTCCCTCTGGTTACAGGAAGC-3′) and reverse (5′-TTCCACCCGTTGCAGCAGGATAGCC-3′) primers and digested with *BsrDI* while *CYP1A1* (m4) was identified from the same 204 bp fragment by using forward (5′-CTGTCTCCCTCTGGTTACAGGAAGC-3′) and reverse (5′-TTCCACCCGTTGCAGCAGGATAGCC-3′) primers using *BsaI* restriction enzyme by 3% agarose gel electrophoresis.

2.3. GSTM1 and GSTT1 genotyping (multiplex - PCR)

GSTM1 and *GSTT1* gene polymorphisms were determined simultaneously using primers (5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′ as well as 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTTCATCCACGTTCACC-3′) and also using internal band forward 5′-ACACAACTGTGTTCACTAGC-3′ and reverse 5′-CAACTTCATCCACGTTCACC-3′ primers by multiplex PCR approach method of Arand et al. (1996). The PCR conditions consisted of an initial single cycle of 10 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 20 s at 59 °C and 5 s at 72 °C and was performed by using the primers of above mentioned. The PCR produced three DNA fragments of 215 bp (*GSTM1*), 480 bp (*GSTT1*) and 350 bp (albumin) used as internal positive control for the PCR efficiency. In both *GSTM1* and *GSTT1* polymorphisms, gene deletions were detected by the existence of null alleles and individuals homozygous with respect to a given null allele lacked the respective PCR amplified DNA fragment.

2.4. NAT2 genotyping

Detection of mutations in *NAT2* gene at 590 G>A and 282 C>T.

First the whole exon region containing variation in the *NAT2* gene was amplified using forward (5'-GTCACACGAGGAAATCAAATGC-3') and reverse (5'-GTTTTCTAGCATGAATCACTC.TGC-3′) primers with specific reagents (Cascorbi et al., 1995). Thermal cycling conditions were 5 min denaturation at 95 °C, then 34 cycles at 95 °C for 30 s, 56.5 °C for 1 min and finally 72 °C for 2 min. This was followed by 5 min extension at 72 °C. The amplified product 1211 bp was determined by using 1% agarose gel electrophoresis.

PCR evaluation of coding region containing nucleotide position at 282 C >T was amplified using 1211 bp fragment as template by forward (5'-GTCACACGAGGAAATCAAATGC-3') and reverse (5'-ACCCAGCATCGACAATGTAATTCCTGCCCTCA-3') primers using suitable reagents (Cascorbi et al., 1995). 1 μl of the diluted PCR product of 1211 bp was added to the template. The thermal cycling conditions used for the amplification was denaturation at 94 °C for 5 min followed by 14 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s. The extension was carried out at 72 °C for 5 min which resulted in the 442 bp