Association between the mitochondrial DNA 4977 common deletion in the hair shaft and hearing loss in presbycusis

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Abstract. The aim of the present study was to examine the role of the mitochondrial (mt) DNA common deletion (CD) 4977 (mtDNACD4977) in the hair shaft in patients with presbycusis. A total of 87 individuals with presbycusis and 95 normal-hearing controls were selected based on strict audiometric criteria. Nested polymerase chain reaction (PCR), sequencing and quantitative (q)PCR were used to examine the expression levels of mtDNA^{CD4977} in the hair shaft in presbycusis. Nested PCR of the hair shaft demonstrated that 8/95 cases with normal hearing were found to be positive for mtDNA^{CD4977}, as compared with 59/87 cases in the presbycusis group. The mtDNA^{CD4977} was positive in 22/43 cases with mild-to-moderate hearing loss, 25/31 cases with moderate-to-severe, severe hearing loss, and 12/13 cases with profound deafness. Statistically significant differences in mtDNA^{CD4977} expression were identified among all of the groups (P<0.001). The sequencing and qPCR assays demonstrated a trend towards an increase in the mean CD level of mtDNA^{CD4977} with a more severe hearing loss at 8 kHz (r=0.778, P<0.001) and all ranges of frequency (r=0.858, P<0.001). In conclusion, the present study demonstrates a correlation between mtDNA^{CD4977} in the human hair shaft and the severity of hearing loss in presbycusis.

Introduction

According to the National Center for Health Statistics, presbycusis is the third most common disease in seniors over the age of 65, following arthritis and high blood pressure (1). The World Health Organization predicted that when there will be ~100 million people aged >60 years, 70-80% of those suffering from presbycusis (2). Presbycusis is characterized by the progressive, bilaterally symmetrical, sensorineural and chronic loss of hearing. Although the mechanism of presbycusis is unclear, Markaryan A et al (3) demonstrated histopathologically, that degeneration of cochlea tissues, including the spiral ganglion, stria vascularis and hair cells, was linearly associated with hearing loss (3).

Numerous studies have reported that deletions of mitochondrial DNA (mtDNA) have a vital role in aging and acquired impairment in various human organs, including the brain, heart, liver, skin, skeletal muscle and cornea (4-6). Cochlea tissue is rich in mtDNA, which have a high probability of deletion. The deletions in mtDNA in cochlea tissue have been shown to include mtDNA 13162, 10422, 7663, 7436, 4989 and 4977 bp deletions, with the mtDNA^{CD4977} being the most common in cochlea tissue (7-12).

Fischel-Ghodsian et al (13) found that mtDNA^{CD4977} was a determining factor in the occurrence and development of presbycusis through polymerase chain reaction (PCR) analysis of temporal bone specimens. In addition, Bai et al (14) reported that mtDNA^{CD4977} was positive in all patients with presbycusis of the same family. mtDNA^{CD4977} in the temporal bone is also associated with the blood supply to the cochlea (15). The diameter of the vessels supplying the internal auditory meatus in patients with presbycusis and mtDNA^{CD4977} has been shown to be less than that observed in patients with presbycusis without mtDNA^{CD4977} (15). Previously, Yamasoba et al (16) demonstrated that mtDNA^{CD4977} aggravates presbycusis in Polg^{D257A} gene knockout mice (16). The rate of mtDNA^{CD4977} increased in Polg^{D257A} gene knockout mice with age-related hearing loss. The acceleration of cochlea degeneration therefore appears to be coupled with mtDNA^{CD4977} (16). These studies indicated that mtDNA^{CD4977} exhibited a close correlation with presbycusis, but the degree of mtDNA^{CD4977} in the hair shaft of patients with presbycusis has not yet been investigated.

The hair shaft expresses a large amount of mtDNA and obtaining the hair shaft is a relatively non-invasive procedure. Analysis of the level and function of mtDNA in the hair shaft may be used to predict the degree of hearing loss.

The present study investigated the significance of mtDNA^{CD4977} expression in the hair shaft in patients with presbycusis and the correlation between mtDNA^{CD4977} and the severity of hearing loss.

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Materials and methods

Case selection. The cases selected for the present study were based on strict audiometric criteria. The criteria were as follows: (i) All of the patients were >60 years old with bilateral sensorineural hearing loss and a downward sloping audiometric pattern; (ii) the hearing loss of all of the patients was symmetrical within 10 dB at each octave; (iii) all of the audiograms in the downward sloping high frequency component had a minimum progression of decreased hearing acuity of 10 dB difference between each successive octave; (iv) hearing loss was defined as a sensorineural threshold ≥30 dB; (v) all of the tympanograms were normal; (vi) the past history was investigated and patients with an identified disease causing hearing loss were excluded (3). A total of 87 cases of presbycusis (between 60 and 83 years of age) who met these strict criteria were selected. Audiograms of 43 cases indicated mild-to-moderate hearing loss. Audiograms of 31 cases indicated moderate-to-severe, severe hearing loss. A total of 13 cases indicated profound deafness. A total of 95 normal hearing individuals were used as control subjects (between 63 and 81 years of age). Between 0.25 and 8 kHz, individuals with normal hearing had bone conduction audiometric thresholds of ≤25 dB. The pure tone averages were analyzed from the bone conduction thresholds at 0.25, 0.5, 1, 2, 4 and 8 kHz. Six hair shafts were collected from each individual (17). Informed consent was obtained from all the patients included in the study, and ethical approval was granted by the Institutional Review Board of Shandong University (Shandong, China).

DNA extraction. Approximately 2 cm hair shaft was cut into pieces and washed three times in 70% alcohol and sterile distilled water. A total of 215 μl 20% Chelex, 10 μl 1 m DTT and 25 μl Proteinase K (18 mg/μl) (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) was added and the hair shaft was incubated overnight at 56˚C until the hair fragments became invisible to the naked eye. The samples were boiled for 8 min, centrifuged at 13,000 x g for 3 min and the supernatant was transferred to a sterile tube and stored at -20˚C.

PCR amplication. The PCR reaction contained 1 μl extracted DNA (40 ng/μl), 0.5 μl each primer (primers 2 and 3, 20 u/μl), 2 μl dNTP (2.5 mm), 0.25 μl Taq enzyme (5 U/μl; Takara, Dalian, China), 2.5 μl buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl) and 18.25 μl sterile distilled H_2O in a volume of 25 μl. The PCR assays were performed on an Mastercycler® gradient (Eppendorf, New York, NY, USA). The PCR reaction condition was as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 60 sec, annealing at 54˚C for 60 sec and extension at 72˚C for 60 sec. The PCR reaction was extended at 72˚C for 7 min. The primers were synthesized by Sangon Biotech (Shanghai, China) (Table I). The products of the PCR amplication was separated by electrophoresis on a 1.0% agarose gel and were detected following staining with ethidium bromide. The images were captured using an Alpha Imager® EC System (Alpha Innotech, San Leandro, CA, USA).

Nested PCR. The nested PCR assays were performed on an Mastercycler® gradient (Eppendorf). The first-step PCR reaction contained 3 μl extracted DNA sample (40 ng/μl), 0.5 μl each primer (primers 4 and 5, 20 u/μl), 2 μl dNTP (2.5 mm), 0.25 μl Taq enzyme (5 U/μl), 2.5 μl buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl), 16.25 μl sterile distilled H_2O, in a volume of 25 μl. The PCR reaction conditions were performed as previously described. A total of 3 μl of the product from the first-step PCR was used as the template in the nested PCR, which used primer 1 and 3 and the same PCR cycling conditions. The products of the nested PCR was separated by electrophoresis on a 1% agarose gel and were detected following staining with ethidium bromide. The images were captured using an Alpha Imager® EC System (Alpha Innotech).

Table I. Primer sequences for mtDNA^{CD4977} amplification.

Primer	Site (nt)	Sequence (5'-3')
1	8292-8312	GCCCACTGTAAAGCTAACTTA
2	13450-13469	TCCCTCACCATTGGCAGCCT
3	13644-13664	CTTCCCCACCCTTACTAACAT
4	8251-8270	GCCCGTATTTACCCTATAGC
5	13845-13826	GTCTAGGGCTGTTAGAAGTC
6	8406-8425	CCCCCATACTCCTTACACTA
7	13525-13506	CGATGATGTGGTCTTTGGAG

The 135-bp fragment may be amplified by primer pair 2 and 3 in normal mtDNA. The primer pairs 4 and 5, 1 and 3, and 6 and 7 are located at the flank of the breakpoint of mtDNA^{CD4977}. Using primers 4 and 5, 1 and 3, and 6 and 7, the 618-, 396-, and 135-bp fragments may be amplified from mtDNA^{CD4977}. mtDNA, mitochondrial DNA; nt, nucleotide.

Sequencing. The amplified fragments were purified and sequenced by BGI-Shenzhen (Shenzhen, Guangzhou, China). The sequenced results was analyzed by Basic Local Alignment Search Tool.

Quantitative (q)PCR. The presence of the mtDNA^{CD4977} was confirmed by sequencing the previously generated PCR product. The PCR assays were performed on a Mastercycler ep realplex (Eppendorf). The PCR reaction contained 3 μl extracted DNA sample (40 ng/μl), 0.5 μl each primer (primers 2 and 3 or primers 6 and 7, 10 u/μl), 12.5 μl SYBR™ Green, 8.5 μl sterile distilled H_2O in a volume of 25 μl. The PCR reaction condition was as follows: initial denaturation at 94˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 15 sec and extension at 68˚C for 30 sec. The melting curve was analyzed at 95˚C for 15 sec, followed by 60˚C for 20 min and terminated at 95˚C for 15 sec. The quantitative variations of mtDNA^{CD4977} were evaluated using the relative cycle threshold (Ct) quantification method (ΔΔ Ct). The relative amount of mRNA was calculated as the calibrator normalized ratio using LightCycler® 480 Software 1.5 (Roche Diagnostics, Mannheim, Germany). The calibrator normalized ratio was measured as the following formula: $RQ = 2^{-\Delta\Delta Ct}$.

Table II. Audiometric thresholds and levels of common deletion across four groups with varying degrees of hearing loss.

Group	Group number, n	Mean age, y	Mean PTA, dB	Mean HL8, dB	Mean CD %
1	22	69±7.3	48.68±10.12	72.72±11.10	11.97±4.12
2	25	72±7.9	51.72±12.27	80.32±15.72	19.75±5.29
3	12	75±8.1	100.00±0.00	100.00±0.00	33.68±10.30
4	7	80±4.2	17.50±5.04	17.50±5.04	4.91±4.16

All cases in the table were positive for the mtDNA^{CD4977} mutation; group 1, cases with mild-to-moderate hearing loss; group 2, cases with moderate-to-severe, severe hearing loss; group 3, cases with profound deafness; group 4, normal hearing. dB, decibels; PTA, pure tone average in dB at 0.5, 1 and 2 kHz; HL8, hearing loss in dB at 8 kHz; CD%, percentage of mtDNA molecules with the common deletion.

Statistical analysis. SPSS version 17.0 was used for the statistical analysis (SPSS, Inc., Chicago, IL, USA). The data are presented as the means ± standard deviation. Analysis of variance (ANOVA) was used to determine whether the differences in the presence of mtDNA^{CD4977} between the different groups were significant. The Pearson correlation coefficient analysis was used to determine the significance of the differences between the presence of mtDNA^{CD4977} and hearing loss. A two-sided P<0.05 was considered to indicate a statistically significant difference.

Results

PCR analysis of mtDNA. The amount of DNA extracted from the hair shaft was in the range of 40-75 ng/μl. In all of the samples of each group, the 135-bp PCR product was detected (Fig. 1A), which indicated the effective extraction of mtDNA from the hair shaft. Through nested PCR, a product of 396-bp was detected in certain samples, which indicated positive expression of mtDNA^{CD4977}. In the age-matched individuals with normal hearing, 8/95 cases (8.42%) were found to have mtDNA^{CD4977} expression by nested PCR. In the presbycusis group, a total of 59/87 cases (67.82%) were detected to have the positive mtDNA^{CD4977} expressed in the hair shaft. Of those 87 cases, the expression of mtDNA^{CD4977} was found in 22/43 cases (51.16%) with mild-to-moderate hearing loss. A total of 25/31 cases (80.65%) with moderate-to-severe, severe hearing loss were indicated to express mtDNA^{CD4977}. A total of 12/13 cases (92.31%) with profound deafness were indicated to express mtDNA^{CD4977} (Fig. 1B). In total, 67.82% of the hair shafts were positive in mtDNA^{CD4977}. Using Pearson correlation coefficients analysis, a statistically significant difference was identified between the presence of mtDNA^{CD4977} and hearing loss (r=0.858; P<0.001). By the use of ANOVA, statistically significant differences of mtDNA^{CD4977} were indicated among all of the groups (F=47.145; P<0.001).

Sequencing of the nested PCR products. The results of the sequencing of the nested PCR products were matched with National Center for Biotechnology Information reference sequence NC_012920 (http://www.ncbi.nlm.nih.gov/nuccore/251831106) in the gene library, which confirmed that the deleted fragment was mtDNA^{CD4977} (Fig. 2). The deleted fragment was located to nucleotides 8470-13447.

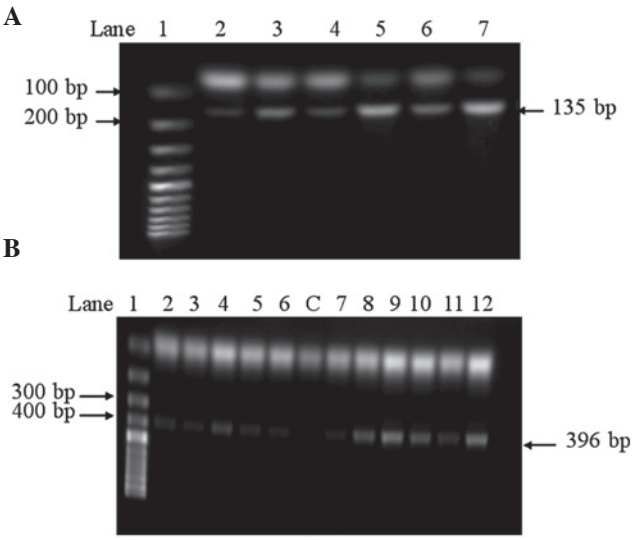

Figure 1. Detection of mitochondrial DNA in the hair shaft. (A) Lane 1, marker; lanes 2-7, 135-bp fragment amplified from normal mtDNA. (B) Lane 1, marker; lanes 2-6, 396-bp fragment amplified from the age-matched control group; lane C, negative control; lanes 7-12, 396-bp fragment amplified from the presbycusis group. bp, base pairs.

qPCR assay. The presence of the mtDNA^{CD4977} was identified by sequencing the generated nested PCR products. The mean level of the common deletion (CD) in the specimens and auditory thresholds of these cases are listed in Table II. A mean CD level of 11.97±4.12, 19.75±5.29, 33.68±10.30 and 4.91±4.16, was detected in groups 1-4, respectively. This difference in CD levels reached statistical significance in all groups (P<0.001). A trend toward increasing levels of the CD with more severe hearing loss was also observed (P<0.001). Furthermore, there was evidence for a significant association between the CD level and hearing loss based on the audiometric thresholds at 8 kHz (r=0.778, P<0.001) and all ranges of frequency (r=0.858, P<0.001).

Discussion

Numerous reports have proposed that oxidative stress may lead to presbycusis. By this hypothesis, oxygen free radicals (OFRs) induce lipid and protein peroxidation, and are considered to damage protein and lipid structure and function. Additionally, OFRs cause deletion and mutation of nuclear and mitochon-

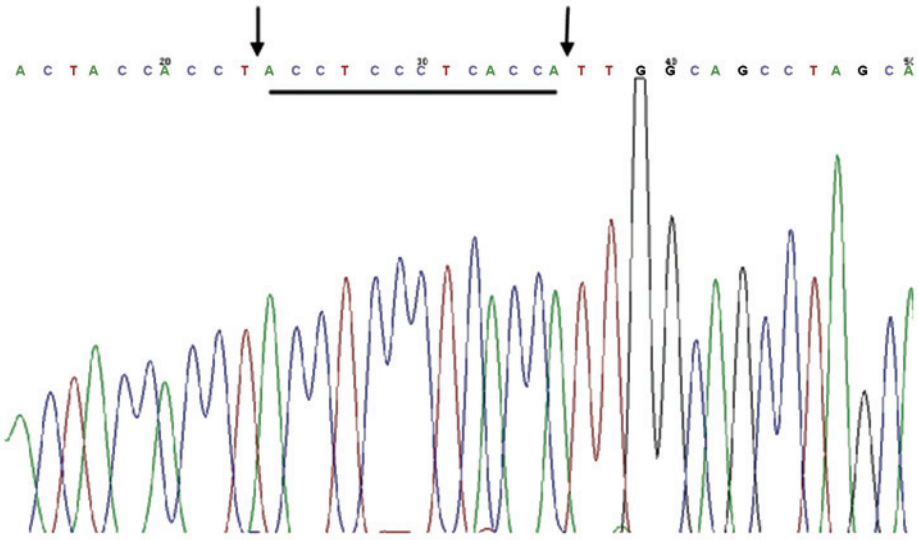

Figure 2. Sequencing chromatogram showing the 4977-bp defect. The left arrow indicates the breakpoint in which a fragment of 4977-bp defect occurs. The sequence between the two arrows is repeats.

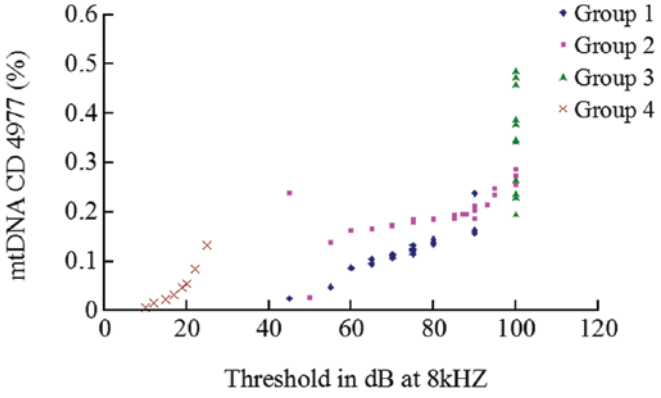

Figure 3. Correlation between the mtDNA^{CD4977} level and the audiometric threshold. Group 1, cases with mild-to-moderate hearing loss; group 2, cases with moderate-to-severe, severe hearing loss; group 3, cases with profound deafness; group 4, normal hearing. A significant correlation between the mtDNA^{CD4977} level and the severity of hearing loss was observed. mtDNA, mitochondrial DNA; CD, common deletion; dB, decibels.

drial genes, which may subsequently cause a significant effect in the ageing and degeneration of cells and tissues (18,19).

Due to a lack in histone protection and defective repair mechanisms, mis-pairing and deletion of highly repetitive sequences in mtDNA occurs frequently under conditions of oxidative stress. The most common mutation or deletion of mtDNA is mtDNA^{CD4977} (20). mtDNA encodes 13 genes, including one cytochrome b gene, two ATP synthetase subunit genes, three cytochrome c genes and seven respiratory chain dehydrogenase subunit genes. These genes are involved in the synthesis of oxidative phosphorylation and mitochondrial function. mtDNA^{CD4977} results in a deficiency of 12 genes, including five tRNA and seven protein-coding genes, which therefore blocks the transcription of complex enzyme I, III, IV and V and oxidative phosphorylation in the respiratory chain (21-23). When generation of ATP through oxidative phosphorylation does not meet the energy requirements of the inner ear, irreversible hearing loss may occur (24,25).

Dai et al (15) examined mtDNA^{CD4977} expression in temporal bone specimens using nested PCR. The authors showed that the percentage of mtDNA^{CD4977} was 50.0% (17/34) in the presbycusis group, 21.1% (4/19) in the age-matched control old-aged group and 0.0% (0/14) in the young and middle-aged group. Markaryan et al (3) studied mtDNA^{CD4977} in cochlear tissues of old-aged individuals. It was found that the incidence of mtDNA^{CD4977} was 32±14% in the presbycusis group and 12±2% in the age-matched normal hearing control group. These studies have therefore demonstrated that mtDNA^{CD4977} is commonly observed in patients with presbycusis. The present study examined the correlation between presbycusis and mtDNA^{CD4977} in the hair shaft. It was identified that the incidence of mtDNA^{CD4977} in patients with presbycusis was significantly higher as compared with the age-matched normal hearing control individuals (67.82% vs. 8.42%, P=0.000). The presented PCR results are consistent with a previous study (7). Furthermore, the PCR products indicated the absence of mtDNA^{CD4977} in positive individuals verified by nested-PCR amplication (Fig. 2). Notably, the degree of mtDNA^{CD4977} is correlated to hearing loss. Based on the qPCR analysis, the degree of mtDNA^{CD4977} in groups 1-4 was 11.97±4.12, 19.75±5.29, 33.68±10.30 and 4.91±4.16, respectively (Table II). To the best of our knowledge, this is the first study to demonstrate the an increase in the percentage of mtDNA^{CD4977} in the hair shaft along with the severity of hearing loss.

Zheng et al (7), detected mtDNA^{CD4977} in the hair shaft of 90 selected cases (age ranges, 5 days-90 years) using PCR. The results indicated that the incidence of mtDNA^{CD4977} expression was 98.3% (89/90). It was therefore concluded that the rate of mtDNA^{CD4977} was correlated with age. The study by Zheng et al differed in conclusion from the majority of the previous reports. These conflicting data may be due to differences in the criteria for selection of the cases, a small sample number and error of detection. Further studies are therefore required to better understand these results. Markaryan et al (3) reported the degree of mtDNA^{CD4977} in the temporal bone was associated with the severity of hearing

loss at 8 kHz (r=0.44, P=0.034; age-adjusted partial correlation =0.55, P=0.007). The present study reconfirmed the a significant association between the $mtDNA^{CD4977}$ expression and hearing loss at 8 kHz (r=0.778, P=0.000) and all ranges of frequency (r=0.858, P<0.001).

The hair shaft has high sensitivity to oxidative stress. As compared with previous in vivo studies (3,15,26), analyzing mtDNA expression in peripheral blood and temporal bone specimens, the present study was non-invasive and effective through analysis of $mtDNA^{CD4977}$ in the hair shaft. Therefore, hair shaft detection may be used to predict hearing function. $mtDNA^{CD4977}$ in the hair shaft may be used as an indicator for the prevention, diagnosis and monitoring of presbycusis.

The present study has demonstrated that $mtDNA^{CD4977}$ in the hair shaft has a close association with presbycusis. Analysis suggested as a method to simplify and further standardise the threshold of $mtDNA^{CD4977}$ resulting in presbycusis reporting.

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