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Original Article

Role of p19ink4d in the pathogenesis of hearing loss

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Abstract: This study aimed to investigate the p19 expression in cisplatin-treated rats and the role of p19 in the degeneration of inner ear cells. It also searched for p19 gene alterations in patients with profound sensorineural deafness. P19ink4d is essential for the postmitotic maintenance of hair cells. It is presumed that a mutation in the functional homolog of p19 or a disturbance in its regulated expression can be the underlying cause of hearing loss. Experiments were conducted on male and female Sprague-Dawley rats (aged 6-7 weeks, 280-320 g) with thresholds of auditory brainstem responses <30 dB in the sound pressure level, and signs of middle ear infection were used for the experiment. For clinical evaluation, 400 children (age less than 13 years) from unrelated families with severe or profound sensorineural hearing loss (SNHL) were recruited at the second Xiangya Hospital of Central South University between 2005 and 2013, and genomic DNA for deafness gene analysis was obtained from peripheral blood samples of the patients and their lineal relatives. It was found that the p19 expression increased over time in the inner ear cells after cisplatin administration, but the p19 mRNA and protein levels significantly decreased in rats with manifested hearing loss induced by cisplatin. However, no mutation existed within the coding exons of p19 in the patients with profound sensorineural deafness. To conclude, the results support the concept that p19 may play an important role in the ototoxic effects of cisplatin and is probably involved in the pathogenesis of hearing loss.

Keywords: Inner ear cells, hearing loss, P19ink4d

Introduction

Hearing loss is one of the most common diseases worldwide. It is an etiologically heterogeneous trait with a variety of genetic and environmental factors, which can be self-existent or co-existent. A genetic factor is estimated to be responsible for about two-thirds of prelingual nonsyndromic hearing loss cases [1]. Of late, more than 150 loci for nonsyndromic hearing loss have been mapped in the human genome, and more than 80 genes have been identified as causative [2].

P19INK4d (hereafter referred to as p19), which is a member of the inhibitors of CDK4 (INK4) family, is a 166-amino-acid protein encoded by the cyclin-dependent kinase inhibitor 2D (CDKN2D) gene located on chromosome 19p13 in humans. CDKN2D is one of the candidate genes within the region of deafness, autosomal recessive 68 (DFNB 68) locus. In p19 knockout mice, sensory hair cells aberrantly re-enter the cell cycle and subsequently undergo apoptosis, resulting in progressive hearing loss [3]. These data emphasize the importance of p19 in active maintenance of the postmitotic state and identify a novel mechanism underlying a nonsyndromic form of progressive hearing loss. Therefore, a mutation in the functional homolog of p19 or a disturbance in its regulated expression can be the underlying cause of hearing loss.

Hearing loss is a common disorder in the general population. However, only a few studies on p19 gene mutation have been performed in patients with hearing loss, and hence to date, no mapped mutations resulting in hearing loss have been found in the coding exons of p19. Therefore, the gene mutation analysis of this locus in patients experiencing hearing loss is warranted.

At present, the level, cell-type specificity, and role of p19 expression in inner ear cells are not well known. A few unique functions of p19 that distinguish it from its siblings have been previously described. Studies suggest that p19 is

very important for the repair of DNA damage [4]. P19 protects cells from undergoing apoptosis by allowing a more efficient DNA repair [5, 6]. Based on these studies, this study proposed that, in addition to its role as cell cycle inhibitor, p19 is also involved in the maintenance of DNA integrity and, therefore, would contribute to the anti-apoptotic function in inner ear cells. P19 is essential for postmitotic state maintenance in hair cells and can act as an anti-apoptotic regulator. The level of p19 expression can determine the sensitivity of hair cells to apoptotic stimuli and result in a predisposition to hair cell loss [3]. Therefore, we propose that p19 may be involved in the degeneration of inner ear cells, resulting in hearing impairment.

To further understand about nonsyndromic hearing loss and its mechanism, this study searched for p19 gene alterations in patients with profound sensorineural deafness. Also, immunohistochemical staining, Western blotting, and real-time polymerase chain reaction (PCR) were performed on cochlear tissues, to elucidate changes in p19 expression in cisplatin-treated rats and examine the role of p19 in the degeneration of inner ear cells.

Materials and methods

Animal procedures

Experiments were conducted on male and female Sprague-Dawley rats aged 6-7 weeks (280-320 g) with thresholds of auditory brainstem responses (ABRs) <30 dB in the sound pressure level (SPL) and signs of middle ear infection on otoscopic examination. Animals were divided into seven groups of five animals each. The animals in all the groups were sacrificed 3 h, 6 h, 12 h, 18 h, 36 h, 1 day, 2 days, and 4 days, respectively, after an intraperitoneal (ip) injection of cisplatin [16 mg/(kg)]. The control group (0-h group) was sacrificed after a single injection of the same volume of sterile saline. All procedures were performed according to the protocols for the care and use of animals approved by the Institutional Laboratory Animal Care Committee of the Second Xiangya Hospital of Central South University.

Auditory brainstem responses

All animals were assessed for their auditory function just before sacrifice. The animals were anesthetized with an ip injection of pentobarbital sodium (35 mg/kg). The auditory brainstem responses (ABRs) were recorded between a subcutaneous stainless steel needle electrode at the vertex and an electrode behind the ipsilateral ear, with a ground electrode on the neck. Stimuli were presented at a repetition rate of 10/s. Around threshold, responses were averaged over 500 stimuli at each intensity level, which varied in steps of 5-dB SPLs.

Tissue collection

The animals were decapitated immediately after the final ABR measurement. The temporal bones were quickly removed and opened to expose the otic capsule. For immunohistochemical staining, the isolated cochlea was fixed in 4% paraformaldehyde for 30 min and then decalcified in 10% EDTA at room temperature with gentle agitation for 6-8 days; the solution was changed every 2 days. The specimens were dehydrated using graded concentrations of alcohol, embedded in paraffin blocks, and then sectioned into 4-μm-thick slices.

For real-time PCR and Western blot, cochleae were microdissected and the cochlear basilar membranes were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

Chemicals and reagents

Polyclonal rabbit CDKN2D antibody (orb100-506), peroxidase-conjugated goat anti-rabbit IgG, diaminobenzidine (DAB), and immunohistochemistry kits (SA1022) were procured from Boster Biological Technology, Ltd, China. Trizol reagent was purchased from Invitrogen Corporation, CA, USA and SYBR Green PCR Master Mix from TaKaRa Biotechnology Limited Company, Dalian, China. The Total Protein Extraction Kit was a product of ProMab Biotechnologies, CA, USA. The bicinchoninic acid protein assay kit was produced by Pierce Biotechnology, IL, USA.

Immunohistochemical staining

After deparaffinization and rehydration, sections were microwaved in 10 mM citric acid (pH 6.8) for antigen retrieval and incubated in 0.3% H2O2 for 10 min to block endogenous peroxidase activity. Then, the samples were incubated with diluted normal goat serum for 20 min to suppress nonspecific binding of immunoglobulin, with rabbit CDKN2D antibody (1:300 dilution) overnight, and then with goat anti-rabbit IgG peroxidase conjugate (1:10 dilution). The

immunostaining signal was visualized after 1- to 5-min incubation with DAB, and sections were counterstained with hematoxylin. In negative controls, the rabbit CDKN2D antibody was replaced with the normal rabbit serum in the reaction. Then, the sections were dehydrated and coverslipped with Permount. The immunohistochemical expression was evaluated with Imag Pro Plus 6.0 (Media Cybernetics, MD, USA) to detect the photo density.

RNA extraction and cDNA preparation

Tissue specimens were directly homogenized in 1 ml of Trizol reagent, and total RNA was isolated. After two chloroform extraction steps, RNA was precipitated with isopropanol, and the pellet was washed twice in 70% ethanol. After air drying, RNA was resuspended in diethylpyrocarbonate-treated water, and the concentration of each sample was determined by measuring the absorbance at 260 nm. Residual DNA was removed using DNase I, in accordance with the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed to cDNA according to the manufacturer's instructions.

Real-time RT-PCR

Real-time reverse transcriptase (RT)-PCR was performed according to the manufacturer's protocol using 4 μl of cDNA and 1 μl of SYBR Green PCR Master Mix (GeneCopoeia, MD, USA). Samples from reverse transcription reactions that did not contain SuperScript III enzyme were used as negative controls. The cDNA abundance for actin was also determined as an internal control. Real-time PCR was carried out on an Eppendorf Master Realplex2 PCR machine (Brinkmann Instruments, NY, USA) using the SYBR Green method. Rat mRNA sequences were obtained from GenBank (Accession: NM_001009719.1) and primers designed using Primer Express 3.0 (Applied Biosystems, MA, USA). The sequences of the primers used are given as follows: for CDKN2D, 5'-GGTCAGCTTCCTAGCTCCTG-3' (sense) and 5'-CATGTGCGACTGCAGAATGT-3' (antisense) and for Rattus actin, 5'-ACTATCGGCAATGAGCGGTT-3' (sense) and 5'-AATGCCTGGGTACATGGTGG-3' (antisense).

All PCR assays were performed as follows: after heating at 95°C for 10 min to inactivate reverse transcriptase, cycling conditions were 95°C for 10 sec, 58°C for 20 sec, and 72°C for 15 sec. Dissociation curves of PCR products were obtained by heating the samples from 60°C to 95°C, to attest to the primers' specificities.

Western blot

Total proteins from the cochlear basilar membrane were extracted using a total protein extraction kit, according to the manufacturer's instructions. Protein concentrations were measured using a bicinchoninic acid protein assay kit. Proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Equal amounts of protein (20 μg) were loaded in each lane of the SDS gel. After transfer to nitrocellulose membrane, bands of CDKN2D were detected using the primary antibody against CDKN2D (1:200). The amount of loading in each lane was verified using a mouse monoclonal antibody (ProMab) as the primary antibody. Secondary antibodies used in the Western blots were goat anti-rabbit and anti-mouse IgG horseradish peroxidase (HRP) conjugate. Bands were detected by an enhanced chemiluminescent substrate and visualized by a Bioshine ChemiQ4600 imaging system (Shanghai Bioshine Scientific Instrument Co., Ltd). Densitometry values were normalized to β-actin immunoreactivity in the same lane to correct for any loading and transfer differences among samples using Image J software, n=3, in each group. All data were triplicated in different times and for different animals.

Subject recruitment and clinical evaluation

A total of 400 children (age less than 13 years) from unrelated families with severe or profound sensorineural hearing loss (SNHL) were recruited at the second Xiangya Hospital of Central South University between 2005 and 2013. Patients in whom DNA microarray and Sanger sequencing identified none mutant alleles of common pathogenic mutation underwent further DNA sequencing. Allele-specific PCR-based universal array (ASPUA) (CapitalBio, Beijing, China) was used to simultaneously screen nine mutations causing hereditary hearing loss (GJB2: c.35delG, c.176 del16, c.235delC, c. 299-300delAT; GJB3: c.538C>T; SLC26A4: c. IVS7-2A>G, c.2168A>G; mtDNA: m.1555A>G, m.1494C>T). Subjects who had been identified by microarray to carry none mutant alleles were further screened for the coding regions of GJB2 gene and 21 exons of the SLC26A4 gene by

Sanger sequencing. Patients in whom DNA microarray identified none of the nine mutant alleles underwent further DNA sequencing.

Informed consent was obtained from all participants prior to the study in accordance with the Institutional Review Board and Ethical Committee of the second Xiangya Hospital of Central South University. Otologists obtained a complete history and physical examination from all subjects. Audiometry was assessed by pure tone audiometry (PTA), ABR, auditory steady-state response (ASSR), immittance testing, and distortion product otoacoustic emissions (DPOAE) according to the age. High-resolution, thin-section CT (HRTSCT) and magnetic resonance imaging (MRI) of temporal bone were performed.

This study was approved by the Ethic Committee of Second Xiangya Hospital of Central South University.

DNA sequencing

Genomic DNA for deafness gene analysis was obtained from peripheral blood samples of the patients and their lineal relatives after informed consent was obtained. Using Primer Express Version 1.0 (Applied Biosystems), primers were designed for the two exons of CDKN2D (EXON1 primers: sense: 5'-AGGAGGGAGGGTGAGTTAGG-3'; antisense: 5'-TCGATCCTCATCCCGCTTAG-3', EXON2 primers: sense: 5'-TGACCCCTTTTGTGGAACCT-3'; antisense: 5'-TGTTTCTTCCCCTCTCTTCTGA-3'). Amplified DNA fragments were purified using a gel extraction kit (Qiagen, Germany), and then sequenced by ABI 377 sequencer (Applied Biosystems, MA, USA). All PCR products were sequenced on both strands and analyzed using DNAstar software (DNASTAR, Inc., WI, USA). The data were compared to GeneBank, RefSeq (CDKN2D, NC_000019.10).

Statistical analysis of data

After real-time PCR, gene amplification was quantified by determining the cycle threshold (CT) based on the fluorescence detected within the geometric region of the semi-log view of the amplification plot. Data were analyzed according to the 2-ΔΔCT method. The ΔCT value was determined by subtracting the target CT of each sample from its respective action CT value. The calculation of ΔΔCT involved using the mean ΔCT value of the control group as an arbitrary constant that was subtracted from all other ΔCT mean values. Fold changes in the expression of the target gene were individually determined. All values were analyzed by one-way analysis of variance at each time point for each treatment, with a significance set at 5%.

Results

Hearing thresholds of ABR

ABR Threshold data at 8.0 kHz were recorded for all the pre-recordings of the eight tested groups. Threshold levels significantly increased 24 and 48 h after the cisplatin injection (37 dB SPL and 70 dB SPL, respectively).

P19 expression in the cochlear membrane

P19 immunolabeling was detected throughout the cochlear basilar membrane, and immunoreactivity was found to be differentially distributed. In the control group, supporting cells exhibited moderate staining and hair cells were lightly immunoreactive (**Figure 1A**). In the 48-h group, the expression of P19ink4d increased, (**Figure 1B**) supporting cells exhibited intense staining, and moderate staining was seen in inner and outer hair cells.

Changes in P19 protein expression

A semi-quantitative analysis of P19 was performed using P19: actin ratio, which was estimated densitometrically; it indicated that the amount of P19 increased significantly 42 h after the ip injection of gentamicin ($P<0.01$); a little peak value was observed at 24 h ($P<0.05$) (**Figure 2**).

Changes in P19 mRNA expression

A decrease in the P19 mRNA expression, which encodes the P19 protein, was reported in the cochlear lateral wall of rats 12-18 h after the ip injection of cisplatin. Also, P19 transcript levels significantly increased 24 h after the cisplatin injection ($P<0.01$) (**Figure 3**).

The change in P19 protein and mRNA expression in cochlea basilar membrane with an irreversible loss of a great extent of inner ear cells

Four days after the ip injection of cisplatin, ABR threshold levels significantly decreased. It can be seen that the expression of P19 in protein

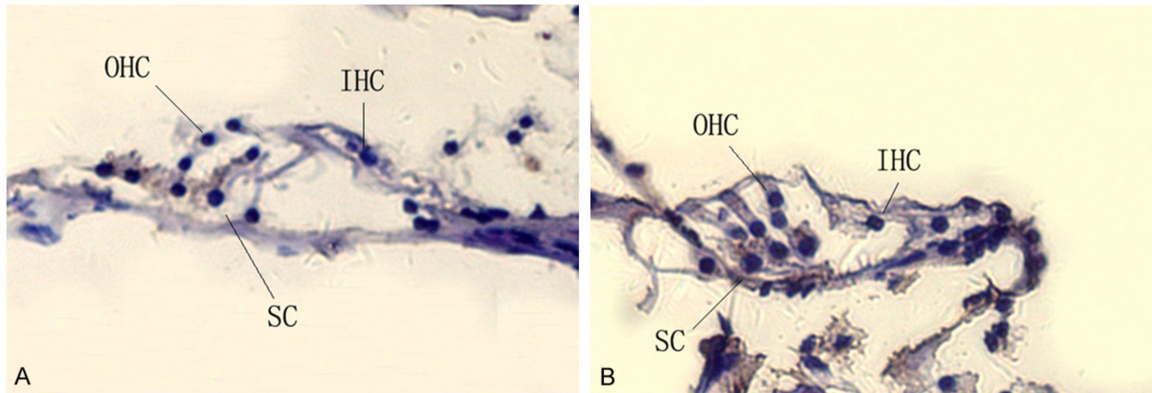

Figure 1. Immunohistochemical staining of P19 (A, B) in rats cochlear basilar membrane. (A) In the control group, supporting cells (SC) exhibited moderate staining and inner hair cells (IHC) were lightly immunoreactive. P19 expression in outer hair cells was negative. Original magnification: 400×. (B) In the 48-h group, the expression of P19ink4d increased, supporting cells (SC) exhibited intense staining, and inner and outer hair cells (IHC and OHC) exhibited moderate staining. Original magnification: 400×.

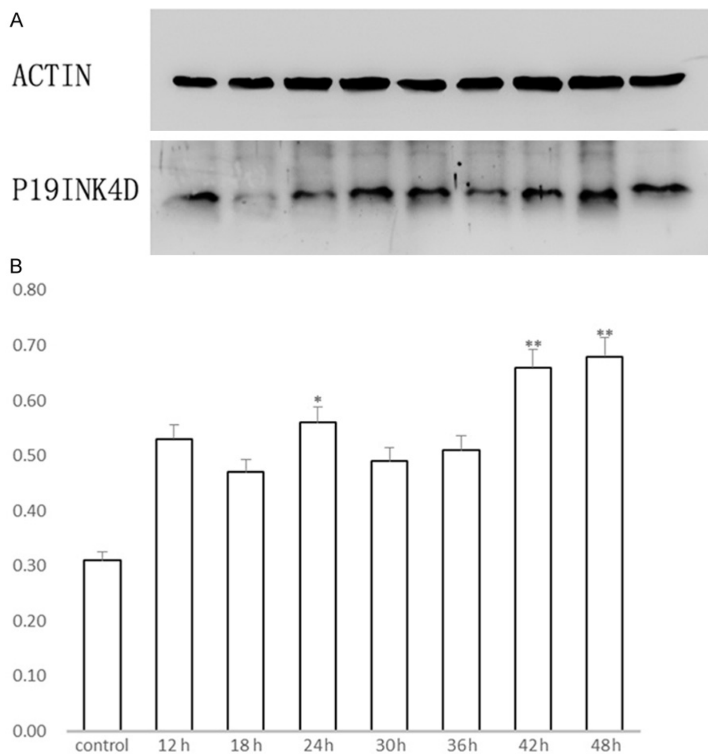

Figure 2. A. Western blotting of P19 protein in the basilar membrane of the rat cochlea. Lower panel: P19; upper panel: internal control (ACTIN). B. Semi-quantitative analysis shows the change in P19 after the administration of a single dose of cisplatin (a significant increase, $P<0.05$ by the one-way ANOVA test, is marked by "*"; $P<0.01$ is marked by "**").

and mRNA level decreased significantly (**Figures 4** and **5**).

Exons of CDKN2D sequence analysis

All 400 subjects could be analyzed successfully and showed a wild-type sequence (**Figure 6**). However, no pathogenic mutation was detected in the coding exons of CDKN2D gene except a synonymous mutation (449 C>G) in exon 2.

Discussion

Outer hair cells (OHCs) are one of the most vulnerable cellular targets of cisplatin action. Other major targets in the cochlea are stria vascularis, supporting cells, inner hair cells (IHCs), and spiral ganglion cells [7]. In the control group, supporting cells exhibited moderate staining and outer hair cells were lightly immunoreactive; no positive reactant was detected in the inner hair cells. In the 48-h group, the expression of p19 increased, supporting cells exhibited intense staining, and moderate staining was seen in inner and outer hair cells. The cell-specific distribution of p19 in the basilar membrane provides morphological evidence that it can play different roles in the development of drug-induced deafness.

Western blot also showed an increase in the p19 expression in 24 h; this increase was significant 42 h after the ip injection of cisplatin. Unexpectedly, the p19 protein levels were not consistent with mRNA levels. Though not statistically significant, an increase in the p19 protein level 12-18 h after the ip injection of cispla-

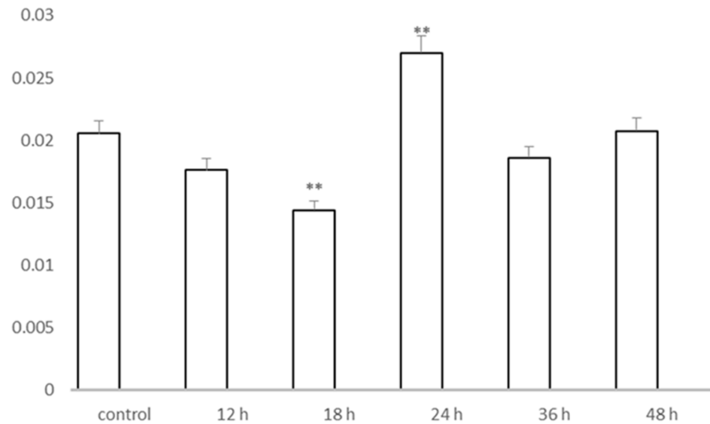

Figure 3. The change in P19 mRNA expression after the administration of a single dose of cisplatin (a significant increase, $P<0.01$ by the one-way ANOVA test, is marked by "**").

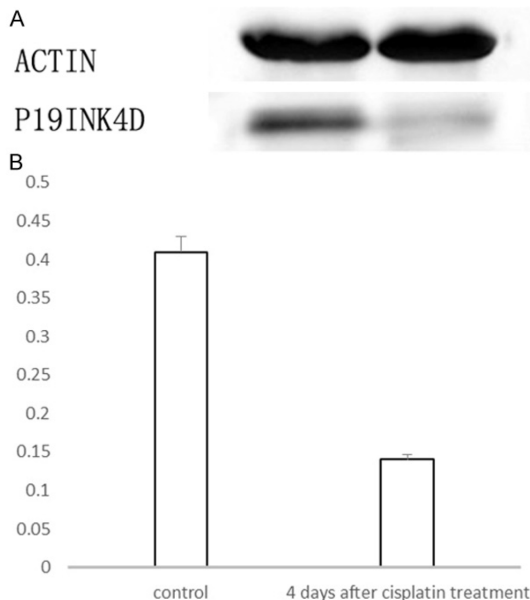

Figure 4. A. Western blotting of P19 protein expression in cochlea basilar membrane. Lower panel: P19; upper panel: internal control (ACTIN). B. Semi-quantitative analysis.

tin, which became significant at 24 h ($P<0.05$). In contrast, there was a tendency toward a decrease in the p19 mRNA expression, which encodes the p19 protein, 12-18 h after the ip injection; no significant increase in mRNA was observed until 24 h. A possible explanation for this discrepancy is that, in 12-24 h after a single-dose cisplatin treatment, the available p19 mRNA is rapidly translated, suggesting that cisplatin has a more rapid effect on the P19ink4d translation/processing than on the transcription of the p19 gene. It indicates that the posttranscriptional mechanism was responsible for the appearance of p19; this needs to be investigated further.

The chemotherapy drug cisplatin is one of the doctors' first lines of defense against malignancies. Cisplatin is cheap and efficacious, but it has a number of side effects that can limit its use, of which nephrotoxicity and ototoxicity are the most serious. Up to 75%-100% of patients receiving cisplatin chemotherapy develop progressive and irreversible SNHL [8]. There are some views on the mechanism of cisplatin ototoxicity, which still have no unified interpretation. The theory of apoptosis is the most popular hypothesis. Cisplatin induces cell death by apoptosis but not necrotic cell death. However, the mechanisms that initiate cisplatin-induced apoptosis are not fully understood. The cell cycle checkpoints play an important role by sensing defects that occur during DNA replication and chromosome segregation, and inducing a cell cycle arrest in response until the defects are repaired [9]. The cell cycle checkpoints are regulated by a family of protein kinases known as the cyclin-dependent kinases (CDKs).

P19 protein, like other INK4s, inhibits CDK4-cyclin D1 activity in vivo and induces G1 phase arrest. The abundance of the transcript of this gene was found to oscillate in a cell-cycle–dependent manner with the lowest expression at mid G1 and a maximal expression during the S phase [10]. Data obtained from sensory hair cells in Ink4d-/- mouse suggest a model in which the maintenance of the postmitotic state of hair cells is dependent on p19 alone [3]. A growing number of studies indicate that cell cycle reentry results in apoptosis by the re-activation of the G1 cell cycle machinery [11-13]. The normal expression of P19 prevents the hair cells from entering an abnormal cell cycle, inducing the cell cycle arrest, which is beneficial for the recovery of damaged hair cells. These data support the notion that p19 is essential for the postmitotic state maintenance of hair cells, and, therefore, acts as an anti-apoptotic regulator.

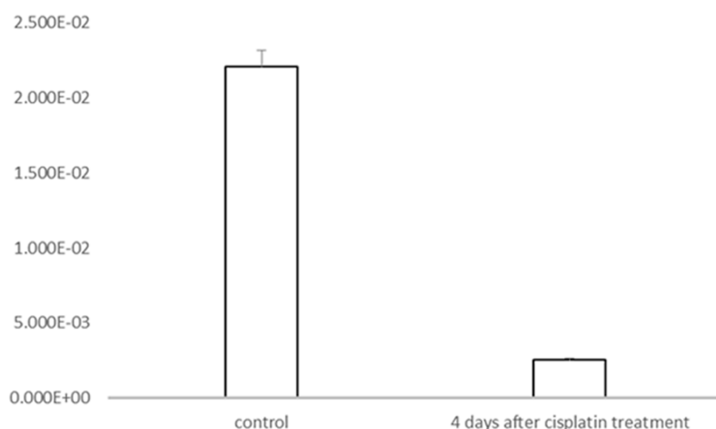

Figure 5. The change in P19 mRNA expression in cochlea basilar membrane.

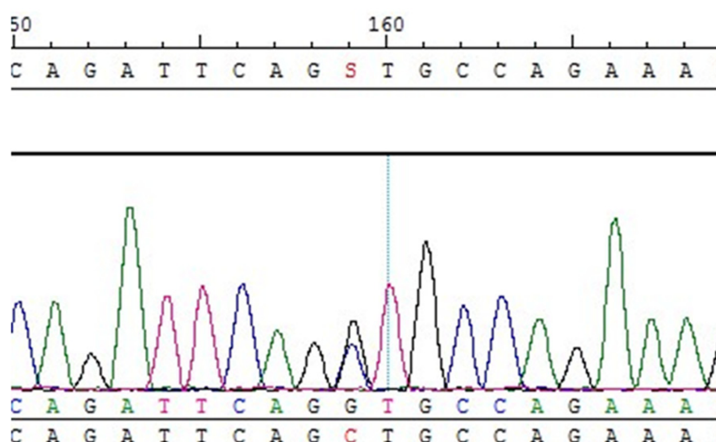

Figure 6. A synonymous mutation (449 C>G) in exon 2 of the CDKN2D gene.

A few unique functions of p19 that distinguish it from its siblings have been reported. In addition to its role in cell cycle regulation, p19 is also important for repairing DNA damage. It has been reported that UV light, cisplatin, and β-amyloid peptide promote p19 transcriptional induction and nuclear translocation [4]. However, the mechanism(s) responsible for p19 upregulation in response to genotoxic stress are yet to be elucidated. Studies suggest that p19 protects cells from undergoing apoptosis by allowing a more efficient DNA repair [14, 15]. More interestingly, p19-underexpressing cells were associated with impaired DNA repair activity, high frequency of chromosomal aberrations, increased apoptosis and, consequently, reduced cell survival when compared with their p19-overexpressing counterparts [5, 6]. p19 activities in DNA repair and cell cycle arrest seem to be independent [14]. Thus, p19 acts as a chromatin accessibility factor that allows the access of the repair machinery to the DNA damage site [15].

In the course of eliminating the damaged cells by triggering apoptosis, a cell first tries to repair any DNA damage and survive. When DNA damage overwhelms the repair capacity, apoptosis occurs. The findings of this study demonstrated that p19 mRNA and protein levels both significantly decreased in the rats with manifested hearing loss 4 days after cisplatin treatment. A previous research by Fang and Hongjun [18] found that the sequences of hair cells were destroyed and hair cell losses were induced 72 hours after treatment with 16 mg/kg (ip) of cisplatin. The research indicated that at this point cisplatin treatment caused an obvious damage to the inner cells, which decreased the cells' ability to maintain and manufacture the normal level of p19. Thus, it is presumed that if there is not yet an irreversible loss of a great extent of inner ear cells, the increased expression of p19 in rat inner ear cells might play an important role in repairing the tissue damage induced by cisplatin. It may participate in detecting DNA damage and directly relay it to the apoptotic machinery. A p19 mutation or a disturbance of its regulated expression can result in the loss of apoptotic control, which in turn may result in hearing loss.

The CDKN2D (cyclic-dependent kinase inhibitor 2D) gene is the human homolog of mouse gene p19. CDKN2D was not found in the inner ear cells, and the level and cell-type specificity of the CKI expression in the human inner ear are not currently known. The gene CDKN2D was screened in two families that had autosomal recessive nonsyndromic hearing impairment (NSHI) from Pakistan but were negative for

functional sequence variants [16]. In this study, CDKN2D was analyzed in 400 samples without any common mutation of hearing loss, but no pathogenic mutation existed in the exons of this gene. The CDKN2D gene seems to be a rare causative gene for profound sensorineural deafness. One reason might be the higher availability of this short gene fragment. Also, animals derived from crosses of Ink4d-null with Kip1-null mice exhibited bradykinesia, proprioceptive abnormalities, and seizures, and died at about 18 days after birth [17]. Patients with a mutation of the CDKN2D gene usually die in utero or in early infancy or have a much more severe brain anomaly and malignancies, in which case it is impossible to carry on the genetic test. The Ping Chen's report showed that hearing is significantly compromised, starting between 7 and 15 weeks of age, and that hearing loss progresses into adulthood as a result of a mutation in the Ink4d gene. These data indicate that a mutation in the Ink4d gene can induce a progressive deafness, rather than a congenital severe hearing loss. The future research should be based on larger sample size or other target groups, such as acquired hearing loss in children.

Conclusion

The systematic administration of a single dose of cisplatin can rapidly influence the expression and distribution of P19 protein and mRNA. Cisplatin has a more rapid effect on the P19 translation/processing than on the transcription of the P19 gene, which suggests an unclear regulation at the posttranscriptional level that requires further investigation. P19 is one of the earliest markers appearing in response to cisplatin, before any toxic effect and cochlear dysfunction. The P19 upregulation is probably one of the common responses of the inner ear cells to injury, such as acoustic trauma and early-stage drug ototoxicity. It may play an important role in the protection and repair of the damaged tissue. When DNA damage overwhelms the repair capacity and the irreversible inner ear cell loss induced by cisplatin occurs, the expression of P19 decreases.

No mutation existed within the coding exons of CDKN2D in 400 patients with profound sensorineural deafness, which suggests only a minor role of CDKN2D gene mutations in congenital severe hearing loss. Expression levels of CDKN2D might influence acquired progressive hearing loss, but the underlying mechanisms need to be explored in more detail.

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Disclosure of conflict of interest

None.

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