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# Reduced expression of Connexin26 and its DNA promoter hypermethylation in the inner ear of mimetic aging rats induced by D-galactose

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ABSTRACT

Connexin26 (Cx26), one of the major protein subunits forming gap junctions (GJs), is important in maintaining homeostasis in the inner ear and normal hearing. Cx26 mutation is one of the most common causes for inherited nonsyndromic deafness, but the relationship between Cx26 and presbycusis is unknown. Our study aimed at exploring the expression and the aberrant methylation of the promoter region of Cx26 gene in the cochlea of inner ear mimetic aging rats. We applied a mimetic aging of inner ear rat model with mtDNA common deletion by D-gal injection for 8 weeks. Real-time RT-PCR and Western blot of rat inner ear tissue indicated that the Cx26 expression decreased in the D-gal group. Further bisulfite sequencing analysis revealed that the methylation status of the promoter region of Cx26 gene in the D-gal group was higher than that in control group. These results indicated that the decrease of Cx26 expression might contribute to the development of presbycusis and the hypermethylation of promoter region of GJB2 might be associated with the Cx26 downregulation.



## 1. Introduction

Age-related hearing loss (AHL), also known as presbycusis, is defined as a progressive, bilateral, high-frequency hearing loss in elderly people [1]. The development of AHL often involves a multistep process which is most likely to be multifactorial [2]. Yet the mechanisms as to how these factors act to cause presbycusis are not fully understood. In order to explore the underlying mechanisms, our lab previously established an animal model through D-gal administration to mimic aging effect on the inner ear of the rats, which was characterized by the presence of the 4834 base-pair (bp) mtDNA deletion, also known as common deletion (CD) [3,4]. The mitochondrial damage caused by reactive oxygen species (ROS) seems to play a central role in the aging process [5]. Overdose of D-gal can be catalyzed into galactitol, which cannot be metabolized in the cells and then lead to osmotic stress and production of ROS, also known as free radical [6]. The production accumulated in the cells may cause mutations/deletions in the mitochondrial genome and sequentially induce mtDNA damage, which then in turn, can lead to decline in ATP synthesis by causing respiratory chain dysfunction and increase in ROS production [7]. This model has been used for the study of pathogenesis of presbycusis [8,9].

Gap junctions (GJs), known as intercellular channels, are one of the most important pathways for the communication of adjacent cells, facilitating the exchanges of metabolite, ion and signaling molecules [10]. Connexin 26 (Cx26), which is encoded by gap junction protein beta-2 (GJB2), is one of the major protein subunits to form gap junctions in the cochlea [11]. Mutations in Cx26 are one of the most common causes of inherited nonsyndromic deafness [12]. Yet, the relationships between Cx26, aging and presbycusis

*Abbreviations:* Cx26, Connexin26; AHL, age-related hearing loss; CD, common deletion; ROS, reactive oxygen species; GJs, gap junctions; GJB2, gap junction protein beta-2.

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remain arcane. In 2000, reduced density of Cx26 staining in the spiral ligament was observed in aging mice [13]. Rodriguez-Paris et al. [14] carried out a genetic analysis of presbycusis and showed that individuals clinically diagnosed with early presbycusis included those with more than one mutation in the GJB2 gene, whereas the control individuals did not. These data suggest that Cx26 may play an important role in maintaining normal hearing, and dysfunction of Cx26 may be related to the development of presbycusis. However, how expression level of Cx26 change and the regulation of Cx26 expression in the inner ear during aging is still unknown.

Methylation of DNA is one of the biologically significant changes occurred in the processing of aging and considered to be one of the programmed aging mechanisms [15]. It was reported that methylation of the cytosine residues at CpG dinucleotides of the promoter region could lead to transcriptional silencing of many genes [16,17]. Because the promoter region of Cx26 gene is rich in CpG dinucleotides, some scholars found that methylation of the CpG sites of the promoter region was an important mechanism in regulating Cx26 expression in human cancers [18]. Here, we investigated the expression level of the Cx26 gene and the CpG site methylation status of the promoter region in the inner ear of mimetic aging rat induced by D-galactose.

## 2. Materials and methods

### 2.1. Animal models

D-Galactose-induced aging rats were established as previously described [4]. Briefly, seven-week-old female Wistar rats were purchased from the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology. After a week of acclimation time, rats were randomly divided into two groups: a control group and a D-gal treated group. They were respectively administrated vehicle (0.9% saline) and D-galactose (500 mg/kg; Sigma, St Louis, MO, USA) daily by subcutaneously injection for 8 weeks. After the last injection, the two groups were continuously feed for 6 months. All rats were housed in an air-conditioned animal facility with a constant temperature of 23 °C and a 12 h light/dark circle. Food and water were given ad libitum during the study. No significant body weight difference was detected after D-gal treatment. All animal experiments performed in this study were approved by the Institutional Animal Care and Use committee at Tongji Medical College, Huazhong University of Science and Technology (Permit No.: S296).

### 2.2. mtDNA common deletion (4834 bp)

The cochleae were dissected immediately from the auditory vesicles upon sacrifice. Total DNA was isolated from cochlea using genomic DNA purification kit (Tiangen Biotech Co., Ltd.) under the manufacturer's instructions. To determine the proportion of mtDNA 4834 bp deletion in the inner ear tissue, the D-loop region copy number of the total amount of mtDNA was measured using TaqMan real-time PCR assay. PCR amplification was accomplished with the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA). The primer and probes sequences for the D-loop region and the mtDNA 4834 bp deletion used in this study have been well described previously [19]. Relative expression level of mtDNA 4834 bp deletion of the experimental group was calculated by the $2^{-\Delta\Delta CT}$ method, with $\Delta\Delta CT$ defined as $\Delta CT_{mtDNA\ 4384\ bp\ deletion} - \Delta CT_{D\text{-}loop}$, indicating the abundance of the mtDNA in each group. The value of control group by definition is 1.

### 2.3. Real-time RT-PCR

Total RNA was isolated from the cochlea, which was dissected out carefully under microscope. The harvested tissue was homogenized in Tri Reagent (Sigma–Aldrich), followed by RNA isolation and purification. A Taqman® Reverse Transcription kit (Applied BioSystems, Inc.) was applied to reverse transcribe mRNA from cochlear tissue into cDNA. After that, quantitative realtime-PCR (qRT-PCR) was performed with a power SYBR green PCR master mix kit (Applied BioSystems, Inc.). The primer sequences for rat beta-actin and Cx26 used in this study were: beta-actin forward: 5′-CGTTGACATCCGTAAAGACCTC-3′; beta-actin reverse: 5′-TAGGAGCCAGGGCAGTAATCT-3′; Cx26 forward: 5′-TCACTGTCCTCTTCATCTTCCG-3′; Cx26 reverse: 5′-CTTCCGTTTCTTTTCGTGTCTC-3′. Relative expression level was calculated for each gene by the $2^{-\Delta\Delta CT}$ method with beta-actin for normalization.

### 2.4. Immunoblotting

Cochlear tissue dissected carefully under microscope was ground in a liquid nitrogen-cooled pestle with a mortar and then lysed in RIPA buffer (Sigma). Equal amount of protein lysates (20 mg/lane) was separated on 12% SDS–PAGE gels, transferred to polyvinylidene difluoride membrane (BioRad, CA), and then blotted with rabbit anti-connexin 26 antibody (Cat. No. 710500, Invitrogen, USA) or chicken anti-GAPDH antibody (Sigma) at 4 °C overnight. After incubation with corresponding horseradish peroxidase-conjugated secondary antibody, the bands were developed by chemiluminescence (ECL; GE Healthcare, Piscataway, NJ, USA) and then visualized by X-ray film exposure [20].

### 2.5. Immunofluorescence

Rats were anesthetized deeply using ketamine (100 mg/kg, i.p.) and chlorpromazine (5 mg/kg, i.p.) before they were perfused transcardially with 4% paraformaldehyde dissolved in phosphate-buffered solution (PBS, pH 7.4). The cochleae were dissected from the auditory vesicles and immersed in 4% paraformaldehyde at 4 °C overnight. After decalcification with 10% EDTA in PBS, they were transferred into 20% sucrose in PBS at 4 °C overnight for dehydration and cryoprotection. The cochleae were embedded in OCT (Sakura, Tokyo, Japan) and cut into sections with a thickness of 10 μm by using a Cryostat (CM3050s, Leica, Germany). The slides were dried in air for 20 min. The nonspecific antigen site was blocked by donkey serum albumin at room temperature for 1 h. Then the samples were incubated with monoclonal anti-Cx26 (diluted 1:100, Cat. No. 138100, Invitrogen, USA) at 4 °C overnight. Fluorescently tagged secondary antibody was used to stain the Cx26 protein for 1 h at room temperature. Then the samples were counterstained with DAPI for 5 min. The photos were taken by the laser scanning confocal microscope (Nikon, Japan).

### 2.6. Bisulfite sequencing

The CpG Island Searcher (http://cpgislands.usc.edu/) was applied to predict CpG site hypermethylation of the promoter region of GJB2 gene (which encodes Cx26) and design bisulfite sequence-PCR primers [21]. Analysis of the hypermethylation status of the promoter region of Cx26 showed two target CpG islands (fragment 1: nt−700 to −483 bp and fragment 2: nt−256 to +81 bp), which contains 10 CpG sites and 29 CpG sites, respectively (Fig. 1 A and B). The primers for amplifying different DNA products after bisulfited were: for fragment 1 forward: 5-TTTTTGGTATTTTGTTTAAAGTGAT-3; reverse: 5-ATATAAACCAACAACTTCCAATATC-3; for fragment 2 forward: 5-GGAGTGATTTAGGTTTTAGGAGAG-3; reverse: 5-TCCCCACAAATCCTAATAAAAACTAC-3. Bisulfite


**Fig. 1.** CpG site of the promoter region of GJB2 gene. Two target CpG fragments (1: nt−483 to −700 bp and 2: nt−256 to +81 bp) of the promoter region of the rat Cx26 gene containing CG pairs (in red) were identified. There were 10 CpG sites in fragment 1 and 29 CpG sites in fragment 2, as shown in the diagrams. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment of genomic DNA extracted from cochlea was well documented previously [22]. Briefly, genomic DNA was extracted with genomic DNA purification kit (Tiangen Biotech Co., Beijing, China) according to the manufacturer's instructions from frozen cochlear tissues isolated under microscope and stored at −20 °C. The purified DNA was bisulfate treated using EZ DNA Methylation-Gold Kit (Zymo, Orange, CA, USA) according to the manufacturer's instructions. Recovered DNA was amplified by PCR using TaKaRa Ex Taq® (TAKARA Bio Inc.). To fractionate cDNA fragments, PCR products were loaded to 1% agarose gel and electrophoresed in TAE buffer for 30 min. Then cDNA were extracted from the gels using E.N.Z.A®Gel extraction kit (Omega, USA) following the manufacturer's instruction. After that, the cDNA were ligated directly into Pmd19 T simple vector (TaKaRa, Dalian, China) at room temperature for more than 2 h according the manufacturer's instructions. The products were used to transform competent *Escherichia coli* DH-5α (TransGen Biotech) and then, the recombinant colonies were selected by LB agar plates containing 100 μg/ml Amp. The consequential recombinant plasmids were then identified by PCR and extracted with TIANprep mini plasmid extraction kit (Tiangen, Beijing, China). The extracted plasmids were sequenced to analyze the status of DNA methylation (Tsingke Biotech Co., Wuhan, China).

### 2.7. Statistical analysis

Data were expressed as means ± sem or absolute value (percentage) where appropriate, with each experiment being carried out independently at least three times. For continuous and normally distributed variables, un-paired Student's *t* test was applied to evaluate the differences between each group. While for categorical value, Chi-square test was used to analyze the statistical difference between each group. Animal number in each group in each experiment varied from 3 to 6. Differences of values of $P < 0.05$ were considered significant.

## 3. Results

### 3.1. The common deletion (CD) incidence increased in the inner ear of aging rats induced by D-gal

We previously established a D-gal injection animal model and demonstrated that D-gal subcutaneous injection could increase the CD mutation rates in the inner ear tissue of rats [4]. In this study the results were consistent with previous finding. D-gal administration increased the CD mutation frequency in rats' inner ear tissue. Compared with the control group, the mtDNA 4834 bp deletion rate of D-gal group was 123.9 ± 13.6%. Continue subcutaneously injection of D-gal significantly increased the CD frequency about 23.87% when 500 mg/kg/d D-gal was given. It demonstrated that the D-galactose-induced aging rats model was successfully established.

### 3.2. The Cx26 expression level decreased in the inner ear of aging rats induced by D-gal

Compared with the control group, the Cx26 protein level and mRNA expression of D-gal treated group was 78.0 ± 8.9% and 66.5 ± 12.0% respectively (Fig. 2A and B). The result indicated that the protein level of Cx26 significantly decreased about 22.0% after D-gal treatment. It is worth noting that, a similar decrease of Cx26 mRNA level, about 33.5%, was also observed by real-time RT-PCR. The immunofluorescent labeling showed that Cx26 was mainly expressed in the spiral limbus, organ of Corti and lateral wall in both NS and D-gal groups. Intensities of immunolabeling did not show any obvious decrease of Cx26 in any part of cochlea (Fig. 2C).

### 3.3. Bisulfite sequencing PCR indicated an increase of CpG site methylation of the promoter region of Cx26 gene in rats receiving D-gal treatment

The bisulfite sequencing PCR was performed to measure the methylation status of the promoter region of Cx26 (including fragments 1 and 2). The CG pairs of fragments 1 and 2 were shown in Fig. 1. Results of bisulfite sequencing analysis for Cx26 gene was shown in Figs. 3 and 4. As the data shown in Fig. 3, D-gal treatment showed hypermethylation of CpG sites in fragment 1 of the promoter region of Cx26 genes, as compared to NS treatment. However, in fragment 2, as shown in Fig. 4, the CpG sites were unmethylated in both D-gal and NS treatment groups.

## 4. Discussion

In this study, we identified that Cx26 was decreased in the inner ear of the mimetic aging rat induced by D-gal, indicating that the function of gap junctions might decline during the aging process.

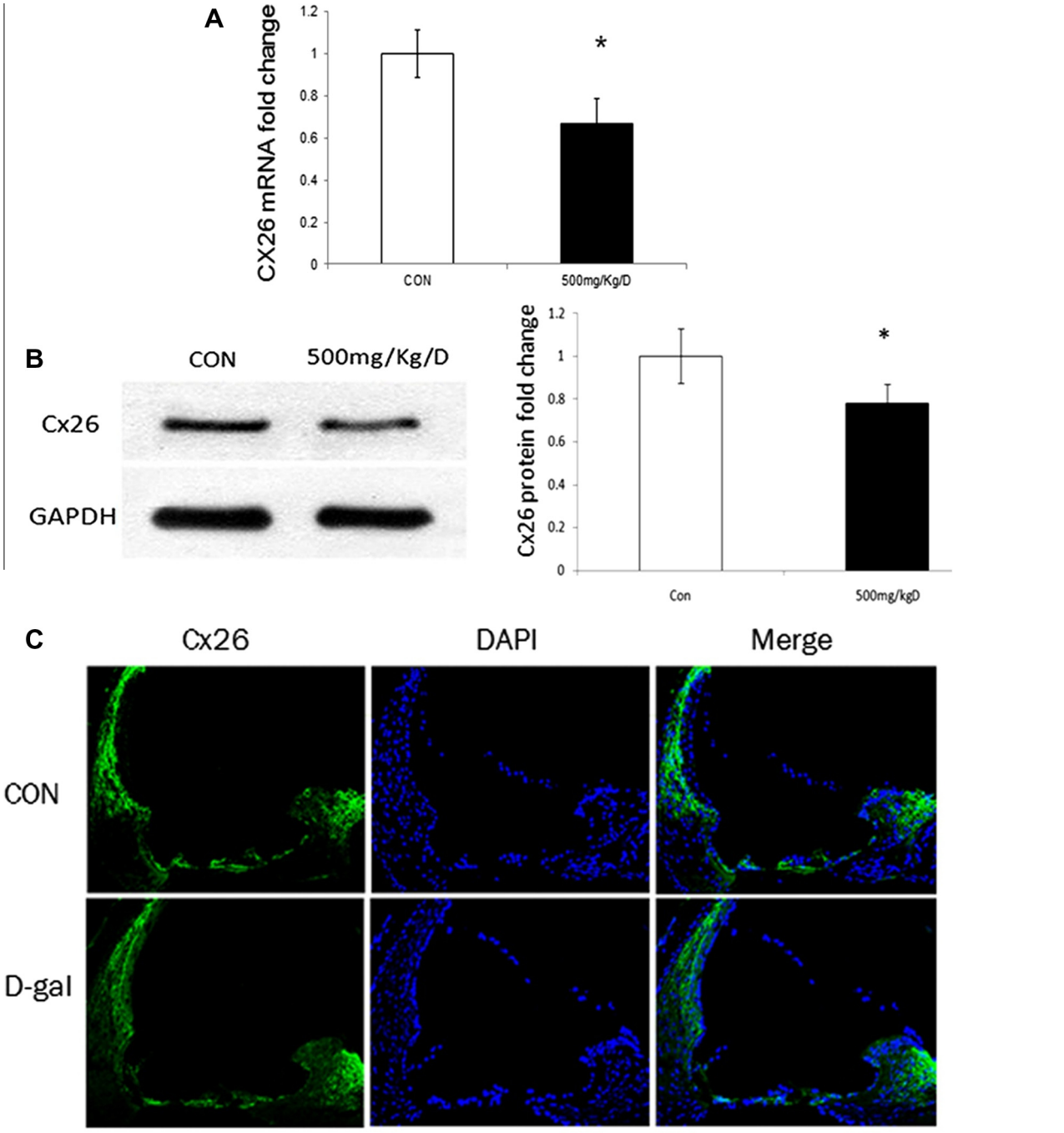


**Fig. 2.** Cx26 expression level in the NS and D-gal treatment groups measured by qRT-PCR, WB and immunoblotting. There were significant decreases of Cx26 mRNA (A) and protein (B) levels in the inner ear tissue when D-gal treatment was given. (C) intensities of immunolabeling did not show any obvious decrease of Cx26 in any part of cochlea. $^{*}p < 0.05$ vs. NS treatment group. $n = 3$ for the NS and D-gal treatment groups.

Cx26, one of the major building blocks of GJs in human cochlea which establishes connectivity in distinct cochlear compartment, is widely expressed in the supporting cells of the sensory epithelial and fibrocytes in the spiral ligament and spiral limbus [23], where it forms hybrid GJs with Cx30 [24]. It plays an essential role in postnatal maturation and homoeostasis of the organ of Corti before the onset of hearing [20,25]. Ahmad et al. [26] over-expressed Cx26 of the cochlea in Cx30-null mice deafness model and found that Cx26 expressed from extra alleles completely restored hearing sensitivity and prevented hair cell death. Consistent with these reports, in the previous study, our lab knocked down Cx26 in mice at the different postnatal time points and showed that Cx26 reduction resulted in hearing loss and cochlear impairment [27]. Chang et al. [28] detailed that deafness linked to loss-of-function connexin mutations was caused by a reduction in the efficiency in the delivery of energetic metabolites (e.g., glucose) through the GJs intercellular network, especially in cochlear regions where microcirculation is less abundant (e.g., the organ of Corti). As a consequence, deficiency in energetic metabolites supply exacerbates ATP exhaustion and ROS generation, which cause accumulative damaging effects to the cellular homeostasis to a level that ultimately lead to cell death and cochlear dysfunction. Given the important role of Cx26 and GJs in the inner ear suggested by these published results, the decrease of the Cx26 level might impair the energetic metabolites supply and contribute to the development of AHL.

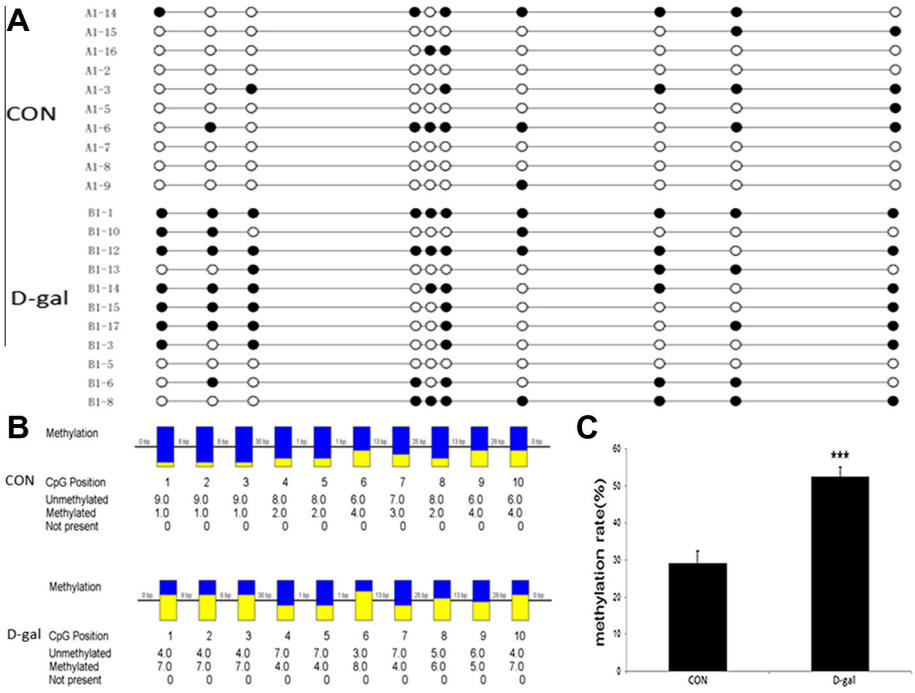


**Fig. 3.** Bisulfite sequencing of fragment 1 after D-gal and NS treatment. Representative sequencing diagrams was showed in (A) and the subsequently summarized data was shown in (B). Methylated ones were indicated as close circles, while unmethylated ones were indicated as open circles. Total methylated CpG rates in D-gal and NS treatment group were displayed in (C), with hypermethylation in D-gal treatment (52.39 ± 2.59%) compared to the NS treatment group (29.02 ± 3.43%). $^{*}P < 0.001$ vs. NS treatment group, $n = 4$.

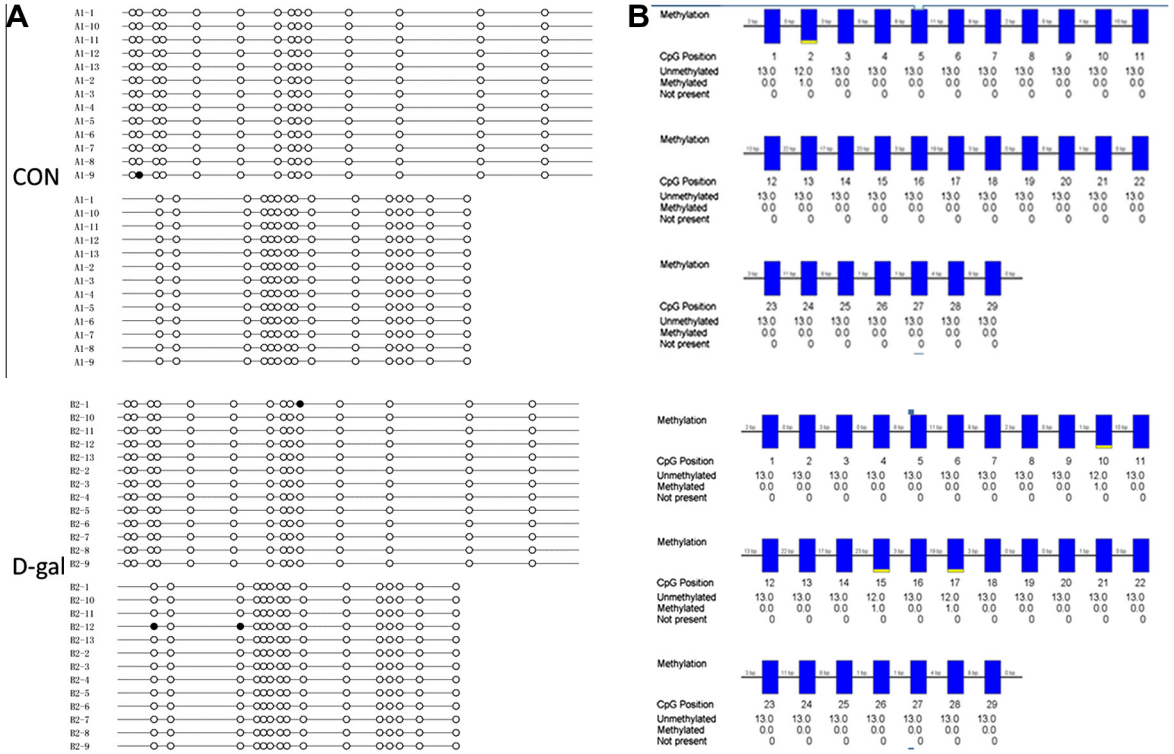


**Fig. 4.** Bisulfite sequencing of fragment 2 after D-gal and NS treatment. Representative sequencing diagrams was showed in (A) and the subsequently summarized data was shown in (B). Methylated ones were indicated as close circles, while unmethylated ones were indicated as open circles. As the data shown, total methylated CpG rates were too low to analyze in both D-gal treatment group and NS treatment group.

In recent years, it has become evident that silencing of Cxs expression may be induced by epigenetic inactivation through aberrant promoter-region methylation [29]. In this research, of the two target CpG clusters that we analyzed, fragment 2 (nt−256 to +81 bp) contains the critical regulatory area (from −97 to −69) which was identified by Tu and Kiang [30], while fragment 1 (nt−700 to −483 bp) located upstream is a novel site identified by this study. Unlike some cancers caused by the hypermethylation of fragment 2 affecting the activation of Cx26, as a tumor suppressor gene [31,32], our results show that the decrease of Cx26 is not caused by the methylation pattern of fragment 2 in D-galactose-induced aging rats. This is similar with the prior study which reported that the hypermethylation of fragment 2 was almost irrelevant to the Cx26 gene repression in human mammary cancer cell lines [33]. In recent reports, methylation of cytosine residues at CpG sites, which are normally unmethylated CG pairs found in GC-rich sequences in the promoter region in the associated gene, was reported to have age-related increases in methylation [34,35]. Our results are similar to those reports. In addition, results showed that the hypermethylation of fragment 1 of GJB2 promoter might be associated with the inactivation of Cx26 in the D-galactose-induced aging group.

It is notable that the promotor-region methylation status of Cx26 in our study is not consistent with it in the previous reports in cancers. The researchers analyzed human full-length cDNA and found that more than a half human RefSeq genes were regulated by putative alternative promoters [36]. Recently, Maunakea et al. [37] highlighted tissue-specific CpG island methylation and a conception of DNA methylation-associated regulation of alternative promoters which could be different in a single cell type in different tissue regions and in different regions of the same gene in the same cell. According to our findings, we speculate that the different CpG clusters in the promoter region of Cx26 gene might lead to different transcription in tissues. This may explain why the methylation status of GJB2 gene promoter was different with the previous studies.

In conclusion, the present investigation suggested that the down regulation of Cx26 might be involved in the development of AHL induced by D-gal treatment in rats and the hypermethylation of fragment 1 in promoter region might be one of the mechanisms. As a result, the down regulation of the Cx26 level in the inner ear leads to the decrease of gap junction mediated intercellular communication. With the decreased energy metabolism and accumulation of ROS induced by the decline of respiratory chain function, which may exacerbate the hypermethylation of CpG site in the promoter region of Cx26, mitochondrial damage would increase to a level that mtDNA4834 bp deletion/mutation developed. To clarify the relationship between the detailed epigenetic regulation of Cx26 and presbycusis, further studies are needed.

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