Development of hair cells in inner ear is associated with expression and promoter methylation of Notch-1 in postnatal mice

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Abstract: The present study was designed to investigate the correlation among the number of hair cells in inner ear, Notch-1 gene expression levels and its methylation status of the promoter region in the postnatal mice. The hair cells in inner ear were collected from postnatal mice at day 0, 4, 8 and 16 and counted by immunofluorescence. Notch-1 mRNA expression were measured by real-time quantitative polymerize chain reaction (PCR). Methylation levels of CpG islands in Notch-1 promoters were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The results showed that the number of hair cells in the inner ear increased gradually after birth, which were positively correlated to Notch-1 mRNA expression. However, analysis on methylation of CpG sites in Notch-1 promoter showed that the methylation rates increased gradually after births, which were correlated with the decreased expression of Notch-1. Drug lesion induced the loss of hair cells, and stimulated the expression of Notch-1 mRNA expression, but didn’t influence the methylation rates of Notch-1 promoter. We concluded that the Notch-1 mRNA expression level in inner ear tissues is correlated with the development of hair cells. CpG islands in Notch-1 promoter region manifest hypermethylation status when hair cells in inner ear are mature.

Keywords: Hair cells, Notch-1, methylation, postnatal mice, inner ear

Introduction

Inner ear hair cells (HCs) are the most important cells for hearing and balance and thought to lack of regeneration after birth. As HCs are injured, auditory function deteriorates. In recent years, deafness caused by hair cells injury increases rapidly because of loud sounds, all kinds of infections, ototoxic drugs, and aging. Previously, only fish and amphibians were thought to have the ability to replace the injured HCs after birth. Humans and other mammals are not easy to replace lost auditory HCs and have limited ability to regenerate vestibular HCs because HCs arising from the embryonic period cannot regenerate after the cessation of mitosis [1, 2]. In mammals, HCs injuries typically lead to the formation of epithelial scars by nearby support cells (SCs) that remain in the epithelium and irreversible hearing and balance deficits. Although there are some trials to stimulate the regeneration of injured HCs after birth, the mechanism of regeneration remains unclear.

The mammalian inner ear contains six separate sensory regions and each sensory region is composed of two kinds of cell types, sensory HCs and associated SCs. Several studies have shown that both cell types arise from a common sensory progenitor cell [3, 4] that differentiates into a hair cell or a supporting cell through lateral inhibition mediated by the Notch signaling pathway [5, 6]. Notch signaling is very important during the development of sensory regions in inner ear, including roles in determining the size of the otic placode [7], in development of the neural and sensory components of the
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inner ear [8, 9], and differentiation of common progenitor cells into HCs and supporting cells through lateral inhibition. Delta-like1 (DLL1) and Jagged 2 ligands expressed in the hair cells are very important signals to mediate the differentiation of HCs in the mouse [10].

A lot of research indicates that HCs replacement does not occur in the mature mammalian inner ear after damage. Two questions are center of interest-What are the reasons and how to stimulate the regeneration of HCs in the inner ear remain highly interested in recent studies. In present study, we investigated the relationship among the number of HCs in inner ear and Notch-1 gene expression levels and its methylation status of the promoter region in the postnatal mice. A mouse model of aminoglycoside-induced deafness introduced by Oesterle also was established to unravel the question as to why mammals have a limited ability to regenerate HCs [11].

Methods

Animals

All Balb/c mice were obtained from the Laboratory Animal Center of General Hospital of Chengdu Military Region. All mice received human care according to the guidelines of the Local Institutes of Health guide for the care and use of laboratory animals. Mice were killed by cervical dislocation or by anesthesia with CO₂ followed by cervical dislocation. Postnatal mice at day 0, 4, 8 and 16 were killed by decapitation.

Drug-lesioning model

According to Oesterle’s model, we injected 1,000 mg/kg of kanamycin and 400 mg/kg of furosemide (intraperitoneal) with an interval of 45 min into the mice to cause the death of HCs. Animals were killed 1 day after the injections. The animals, only injected with saline, were used as controls [11].

Immunofluorescent staining

Cold 4% paraformaldehyde was used to fix the cochlea for 4 h at room temperature. Segments of the organ of Corti were carefully dissected free from the cochlea and rinsed in PBS, and sequentially infiltrated with sucrose (10% for 30 min, 15% for 30 min, 1:1 mix 15% sucrose/0.15% overnight) and 0.1% Triton X-100. Alexa 594-labeled secondary antibodies (Invitrogen/Molecular Probes) were used at a dilution of 1:250 in PBS with bovine serum albumin and Triton X-100. After counterstaining nuclei with propidium iodide (Invitrogen Corp, USA), specimens were mounted in 30% glycerol and examined with epifluorescence.

Cell collection

The cochlea was exposed by open otocyst under a dissecting microscope in Dulbecco phosphate-buffered saline DPBS solution. Then the cover film and vascular pattern were removed and the cochlear sensory epithelium was separated with a sharp needle. The sensory epithelium was placed in PBS containing 0.125% trypsin and 0.125% collagenase at 37°C 8 min. Then the digestion was terminated by pipeting 20 times with a 1 ml of straw and next added DMEM with 10% fetal bovine serum (FBS). The cells were observed under the phase contrast microscope (Nikon, Japan).

Isolation of total RNA and real-time PCR

Total RNA was extracted from the tissue samples using TRIzol agent and cDNA was prepared according to the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) instructions. Reference gene was GAPDH. Target genes were relatively quantified by Syber green dye method. Primers of Notch-1 ‘gene is 5'-TGGCTCTCAATGGGATACAAATG-3’ and 5'-GGGCAACACACCTAC-3'. RT-PCR reaction conditions were as follows: 95°C for 2 min, 72°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 30 s. The fluorescence signal was acquired during the extension step at 60°C. A melting curve of the reaction system was drawn immediately after the reaction to analyze the specificity of the PCR products. Quantitative analysis of target gene expression data was based on the 2^ΔCt method.
DNA methylation

The Notch-1 gene sequence was obtained from GenBank and the positions of the gene promoters were predicted using the Promoter 2.0 Prediction Server at http://www.cbs.dtu.dk/services/Promoter and www.PromoterScan at http://www-bimas.cit.nih.gov/molbio/proscan. The MethPrimer primer design website was used to predict the CpG islands. Cellular genomic DNA was extracted using the BioTek DNA purification kit (BioTek, Beijing, China). For each sample, a total of 200 ng of genomic DNA from each sample was bisulfite-treated using the EZ-96 DNA methylation kit (Zymo Research) according to the manufacturer’s instructions. The genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracil. Sequenom MassARRAY platform (CapitalBio, Beijing, China) was used to detect Notch-
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1 gene promoter methylation, quantitatively. This platform was composed of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and combined with RNA base-specific cleavage. Specific primers for methylation measurement were designed using Epidesigner (Sequenom, San Diego, CA, USA) as belows: 5’ TTTGTGTTTTTTGGGGTTATTT 3’ and 5’ CCTAACCCAACRACTTTCACT 3’ (302 bp). PCR was performed in a thermocycler (Eppendorf Mastercycler gradient, Germany). Mass spectra of PCR products were obtained via MassARRAY Compact MALDI-TOF (Sequenom) and their methylation ratios were generated using the Epityper software version 1.0 (Sequenom).

Statistical analysis

Statistical analysis was performed using the SPSS statistical package version 14.0 (SPSS, Chicago, IL, USA). Descriptive results of continuous variables are expressed as mean ± Standard Deviation (SD) for normal distribution variables. Means were compared by 2-way Analysis of Variance (ANOVA). Medians were tested by Mann-Whitney rank sum test. The level of statistical significance was set at 0.05.

Results

The number of hair cells in inner ear

Parvalbumin antibody is a kind of antibody used to label HCs in mice and other mammals previously. Parvalbumin is a Ca2+ -binding protein also observed in nerve terminals, nerve fibers, and spinal ganglion cell bodies. By using immunofluorescent staining, we observed that the auditory epithelium is composed of inner and outer HCs (Figure 1A). The inner HCs keep stable, while the outer HCs still grow slowly. Statistical results showed the outer HCs after birth were 18 per 10 μm, and increased gradu-

Figure 3. Quantitative DNA methylation of CpG islands in Notch-1 promoter region. A. The represent image of methylation assay. B. The average methylation rates of CpG islands. C. The correlation of CpG site methylation rates and mRNA expression of Notch-1. D. The correlation of CpG site methylation rates and the increased number of outer HCs.
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ally and the increased number of HCs decreased with the time (Figure 1B).

Notch-1 mRNA expression

Real-time PCR results showed that Notch-1 mRNA abundance were significantly decreased from postnatal day 0 to day 16 in a time-dependent manner (F = 3.897, P = 0.007) (Figure 2A). Correlated analysis results showed the decrease of Notch-1 mRNA expression was positively associated with the decreased growth rates of HCs after birth (Figure 2B).

Methylation analysis of Notch-1 promoter region

Human Notch-1 sequences were searched in GenBank. The length of CpG island was 302 bp (955 bp to 1256 bp). The island contained a total of 33 CpG sites. Agarose gel electrophoresis of bisulfite sequencing PCR amplification products showed a single desired product of the same size as the expected target fragment. MALDI-TOF MS analysis detected that CpG islands in Notch-1 promoter showed a hypermethylation status in the growth process after birth. The average methylation rate of CpG island increased with time after birth (P<0.001, Figure 3A, 3B). Correlated analysis results showed the decrease of Notch-1 mRNA expression was associated with the increase of CpG site methylation (Figure 3C). Correlated analysis results also showed the decreased growth rates of HCs was associated with CpG site methylation (Figure 3D).

Influence of drug-lesioning HCs on notch-1 expression and CpG site methylation

We used the HCs-specific antibody parvalbumin to analyse HCs loss. The results verified that the loss of a lot of outer HCs at 1 day after the injections (Figure 4A). The loss of single-labeled HCs and nuclei in the HCs layer didn’t influence the CpG site methylation of Notch-1, but induced the mRNA expression of Notch-1 (Figure 4B, 4C).

Discussion

The inner ear is the innermost part of the vertebrate ear and is an intricate organ designed to transmit both auditory and balance information. The development of the inner ear involves dramatic morphogenetic and patterning events
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which mold its shape from a simple thickened epithelium or placode, to a complex membraneous labyrinth complete with semicircular canals, a coiled cochlea, and six sensory areas innervated by afferent neurons that also derive from the placode. Each sensory organ is composed of two basic cell types, the sensory hair cell and non-sensory supporting cells arranged in an invariant and alternating mosaic in which each hair cell is surrounded by supporting cells. A lot of research indicated that the patterning of the sensory regions could be achieved through cell-cell interactions mediated by the Notch signaling pathway [12, 13]. The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms. Notch regulates the development of most organs and tissues, and the adult maintenance of stem cells and tissues. Mutations in the core pathway can lead to developmental disorders, adult diseases, and cancer [14, 15]. Notch1 expression is specific to hair cells and Jagged 1 to supporting cells. In the inner ear, evidence has accumulated that Notch signaling plays a crucial role in the differentiation of hair cells, supporting cells and neurons in the developing inner ear epithelium in both mammalian and non-mammalian vertebrates. Notch signaling is activated by Notch ligands (Delta or Serrate/Jagged family) binding to the Notch receptor in adjacent cells, which leads to γ-secretase mediated cleavage and generation of Notch intracellular domain (NICD) fragments. NICD translocates to the nucleus and stimulates expression of two inhibitory basic helix-loop-helix proteins, hairy and enhancer of split 1 (Hes1) and Hes5, which down-regulate the expression of prosensory genes such as Atoh1, an essential regulator of hair cell development. This in turn leads to the inhibition of hair cell fate and the development of the cell as a supporting cell. Considering evidence that notch signaling pathway serves vital roles before birth, the expression of Notch signaling after birth is unclear. Based on our findings, after birth, the growth of HCs increased gradually and the increased number of hair cells was positively correlated to Notch-1 mRNA expression. Methylation of CpG site in Notch-1 promoter also increased by time after birth, which was correlated with the decreased expression of Notch-1 mRNA expression, demonstrating that Notch signaling is very important on the growth and maturity of HCs in the inner ear after birth.

By experimenting drug-lesioning model, we observed that loss of HCs didn’t lead to demethylation change of Notch-1 promoter, but activated the expression of notch-1 mRNA expression, indicating that Notch-1 may be regulated by many other factors such as microRNAs and transcriptional factors within a certain range [16, 17]. Activation of Notch-1 leads to ectopic sensory progenitors that differentiate into hair cells and supporting cells [18], so it may be related with the wound repair of HCs. Taken together, the drug-lesioning studies indicated that Notch-1 is required for the initiation of growth of HCs; however, the ability to regenerate is limited.

This study explains the key role of Notch 1 signaling pathway after birth. This study, for the first time, explored the Notch-1 DNA methylation status on Notch-1 promoter and its relationship with the development of hair cells in inner ear and Notch-1 mRNA expression in mice after birth. Our data showed that Notch-1 promoter was hypermethylated in all subjects after 16 days and remained unchanged until adults or after injuries. To assess the association between Notch-1 CpG methylation rates and the development of hair cells in inner ear, we performed correlation analysis. Results showed that CpG methylation rate seems correlated to the development of hair cells in inner ear.

In conclusion, these findings are interesting and show that Notch-1 is very important in the development and maturity of hair cells. Thus, Notch-1 might be an important pathway to manipulate when designing therapies to treat deafness and balance disorders caused by loss or dysfunction of HCs.

Disclosure of conflict of interest

None.

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