TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2017) 47: 1626-1633

doi:10.3906/sag-1604-137

DNA methylation of the prestin gene and outer hair cell electromotile response of the cochlea in salicylate administration*

Erdoğan BULUT[1,2,], Metin BUDAK[3], Levent ÖZTÜRK[2], Mehmet T. TÜRKMEN[4], Cem UZUN[5], Tammam SİPAHİ[3]**

[1]Department of Audiology, Faculty of Health Sciences, Trakya University, Edirne, Turkey
[2]Department of Physiology, Faculty of Medicine, Trakya University, Edirne, Turkey
[3]Department of Biophysics, Faculty of Medicine, Trakya University, Edirne, Turkey
[4]Department of Otorhinolaryngology, Samsun Training and Research Hospital, Ministry of Health, Samsun, Turkey
[5]Department of Otorhinolaryngology, Faculty of Medicine, Trakya University, Edirne, Turkey

Received: 28.04.2016 • **Accepted/Published Online:** 04.07.2017 • **Final Version:** 13.11.2017

Background/aim: Activity of the prestin gene may have a role in the pathogenesis of salicylate-induced ototoxicity. We investigated DNA methylation for prestin gene exon 1 in salicylate-injected guinea pigs.

Materials and methods: Fifteen guinea pigs (30 ears) underwent audiological evaluation including 1000 Hz probe-tone tympanometry and a distortion product otoacoustic emission (DPOAE) test. The animals were randomly divided into three groups. Groups 2 (8 ears) and 3 (14 ears) were injected with intramuscular saline and sodium salicylate (200 mg/kg), respectively twice daily for 2 weeks. Group 1 (8 ears) received no injection. DPOAE measurements were performed at baseline; after 1, 2, 4, and 8 h (acute effect); and after 1 and 2 weeks (chronic effect). After audiological measurements, the animals were sacrificed for DNA isolation.

Results: While a significant decrease ($P < 0.01$) was found for the acute effect in all frequencies in Group 3 according to baseline measurements, there was no difference in terms of chronic effect. DNA methylation increased during the acute phase of salicylate administration, whereas it returned to initial levels during the chronic phase.

Conclusion: Salicylate-induced changes in DPOAE responses may be related to prestin-gene methylation. These results may have important implications for salicylate ototoxicity.

Key words: Ototoxicity, tinnitus, hair cells, auditory, outer, distortion product otoacoustic emission, sodium salicylate, DNA methylation

1. Introduction

Outer hair cells (OHCs) change their length in response to membrane potential alterations (1). Outer hair cell (OHC) motility appears to amplify basilar membrane vibration actively, a process often referred to as cochlear amplification (2). A transmembrane motor protein called prestin has been described in the lateral wall of OHCs (3). Prestin uses cytoplasmic anions (mainly Cl^-) as extrinsic voltage sensors and changes OHC length in response to the alterations in membrane potential (3,4). These anions (especially Cl^-) bind to prestin with millimolar affinity and move towards the intracellular region with changes in membrane potential while OHC is in the depolarization phase, leading to shortening of cell length, whereas they move towards the extracellular region during hyperpolarization, resulting in increased cell length. When there is no monovalent anion in the cytoplasm, the prestin molecule remains in short form with OHC in maximal contraction (3,5). The monovalent anion affinity of prestin is as follows: $I^- > Br^- > NO^-_3 > Cl^- > HCO^-_3 > F^-$ (3). In a recent study, prestin-based OHC motility was demonstrated to be necessary for mammalian cochlear amplification, and prestin gene knockout animals showed reduction in distortion product otoacoustic emissions (DPOAEs) and hearing loss (5,6). Oliver et al. (7) have shown that salicylate, the active component of aspirin, binds to the anion (Cl^-) binding domain of prestin as a competitive antagonist and this binding domain has 300-fold affinity for salicylates compared to Cl^- anions. In addition to its blocking effect on prestin, salicylate may

* This study was presented as an oral presentation in the 36. National Congress of Turkish Society for Otorhinolaryngology & Head Neck Surgery, Antalya, Turkey (5–9 November 2014).

** Correspondence: erdoganbulut@gmail.com

affect the mechanical properties of the stereocilia bundle by decreasing its bending stiffness (8). Accordingly, several studies have demonstrated decreased OHC motility with high-dose aspirin intake, which may result in hearing loss (9,10). Salicylate administration may cause tinnitus in real life and some studies suggest that tinnitus may be considered an auditory nerve dysfunction (11). Tinnitus is a commonly encountered symptom in hearing disorders, similar to pain that accompanies several conditions (12,13). Therefore, this study may have important implications for tinnitus, which is associated with a hearing loss of known origin in 90% of cases (12–14). Tinnitus often coexists with cochlear damage (15). Tinnitus is thought to develop as a result of the spontaneous increase in firing rates of primary auditory nerve fibers caused by hair cell hyperactivity (16). While some studies support the role of NMDA receptors in cochlear excitotoxicity-induced tinnitus (11,16), the relevant molecular mechanisms remain unclear. Gene expression studies in salicylate-induced experimental tinnitus models have not demonstrated increased NMDA receptor expression at transcriptional level in the cochlea, whereas an increase has been observed in prestin gene expression (17), which is the motor protein responsible for increased electromotile responses in OHCs (18,19). Studies have been limited to the cochlea, and the molecular factors that may have significant effects on the cochlea have not been comprehensively evaluated. Recent studies that investigated the influence of age, developmental factors, and environmental factors (stress etc.) on nucleotide sequences demonstrated gene silencing by methylation, which causes only chemical changes in DNA in conditions such as carcinogenesis while unmethylation has been associated with gene activation (20,21). We aimed to investigate OHC electromotile responses with acute and chronic salicylate administration and potential epigenetic modifications in exon 1 of the prestin gene by means of methylation-specific polymerase chain reaction (MSP).

2. Materials and methods

2.1. Animals

After approval was obtained from the local ethics committee (Animal Care and Use Committee of Trakya University with protocol number 2013/27), 15 healthy young-adult pigmented guinea pigs (weight range, 200–320 g) with normal auropalpebral reflexes were included in the study. All animals underwent bilateral otoscopic examination and audiological evaluation including 1000 Hz probe tone tympanometry and DPOAE tests. Tympanometric and DPOAE measurements were performed with a Madsen Capella (GN Otometrics A/S, Denmark) OAE device. The probe assembly was fixed with an adaptor in order to allow tight and deep insertion into the ear canals of guinea pigs, and the probe was fixed in the appropriate position with a holder during measurements. The animals were placed in a sound-insulated chamber. All measurements were performed when the guinea pigs were under general anesthesia achieved by sodium pentobarbital (40 mg/kg). Body temperature was maintained at 38 °C with a warming blanket.

2.2. Salicylate administration

According to Jastreboff's tinnitus model (22–24), sodium salicylate (200 mg/kg) was administered by intramuscular (i.m.) injection twice daily for 2 weeks. Sodium salicylate was purchased from Sigma (St. Sigma-Aldrich; Germany) and was freshly dissolved in normal saline to obtain a final concentration of 200 mg/mL.

2.3. Study protocol

Fifteen guinea pigs (n = 30 ears) with normal findings in tympanometry and DPOAE tests were randomly divided into three groups: Group 1 served as the home-cage control, Group 2 served as the placebo control, and Group 3 served as the active drug (salicylate) group.

Group 1 (negative control group, n = 8 ears): No drug administration was performed for the guinea pigs in this group. Group 2 (positive control group, n = 8 ears): The guinea pigs in this group were administered two doses of normal saline (NaCl 0.9%) 200 mg/kg per day by i.m. injection for 2 weeks. Group 3 (experimental group, n = 8 ears): Two doses of salicylate 200 mg/kg per day were administered by i.m. injection to the guinea pigs in this group. Audiological evaluations for chronic effect after sodium salicylate or normal saline injections were performed on day 0, week 1, and week 2. DPOAE was recorded before and 1, 2, 4, and 8 h after the injections to assess the acute effect. For evaluation of the acute (0 to 8 h) and chronic (8th hour to 2 weeks) effects of salicylate administration, the guinea pigs were decapitated under high-dose anesthesia (200 mg/kg sodium pentobarbital) and their temporal bones were dissected. Cochleae were removed, DNA isolation was performed, and the isolates were subjected to bisulfite modification followed by gene methylation analysis employing MSP with methylation-specific and unmethylated DNA primers for exon 1 of the prestin gene. This study comprised audiological evaluation and molecular analysis.

2.4. Audiological evaluation

2.4.1. Tympanometric examination

The probe was set at 1000 Hz, the pump speed was 100 daPa/s, and the pressure range of measurement was set at +200 daPa to –200 daPa. Type "A" tympanograms (peak pressure: from +100 daPa to –100 daPa) were accepted as normal.

2.4.2. DPOAE measurements

Acoustic stimuli were two continuous pure tones at the so-called primary frequencies, f1 and f2. Primary levels L1

and L2 were adjusted separately and their frequency ratio f2/f1 was fixed at 1.22. Stimulus levels were fixed at L1 = 65 and L2 = 55 dB SPL. DPOAE measurement was evaluated when 2f1 - f2 DPOAE was generated by primaries with geometric mean frequencies between 0.75 and 8 kHz. Testing time was 60 s. A common criterion to confirm detection of the DPOAE was its amplitude being at least 3 dB above the average level of the noise floor sampled at several frequencies surrounding the emission frequency (25). Frequency-specific signal/noise ratios (S/N-R) were observed in both ears of the guinea pigs.

2.5. Molecular analysis

2.5.1. Cochlear DNA isolation

For acute (the first 8 h) and chronic (8th hour to 2nd week) effects, the guinea pigs were decapitated under high-dose anesthesia and their temporal bones were dissected. The temporal bones were placed in liquid nitrogen in order to minimize surrounding tissue contamination. The otic capsules (Figure 1) were dissected in liquid nitrogen and cochlear materials were homogenized. A GeneMATRIX bone DNA purification kit was used for DNA isolation from the cochlear structure.

2.5.2. Methylation-specific polymerase chain reaction

DNA purity was measured at 260 and 280 nm wavelength on spectrophotometry and DNA quantification was performed using the DNA (μg/mL) = A260 × Dilution Factor × 50 (coefficient) formula at 260 nm UV. Next 10 μL of DNA was taken from each sample and bisulfite modification was carried out for DNA according to the Millipore CpGenome modification kit and manuscript. This modification converts the cytosines in the unmethylated region to thymine. For the region thought to be altered in this manner, CpG sequences in exon 1 of the prestin gene were detected using the MethPrimer V1.1 beta program (26). MSP was conducted by means of the following protocols in order to investigate methylation.

Figure 1. Following the dissection of the temporal bone, the otic capsule and cochlea were exposed. The cochlea was removed entirely and used for molecular analysis, i.e. DNA isolation and methylation assay (magnification ratios: 15 × 1.5 × 11.2).

PCR conditions for the methylated and unmethylated regions,

PCR primers for exon 1 of prestin, methylated region (M: methylated):

MForward: ATGTTGAAGAAAATGAAATTTTCGT,

MReverse: ACTTATCCCCGATAAAATCCG, PCR product: 164 base pairs (bp),

Unmethylated region (UnM: unmethylated):

UnMForward: TTTATTTTTAGAAGGTTGTGG,

UnM-Reverse: AAACTACCAAACAAAAAACAACATC, PCR product: 163 bp.

PCR conditions: PCR buffer 1x, MgCl: 2 2.5 mM, DMSO: 5% (v/v), dNTP: 12.5 mM, Primer Forward: 10 pmol, Primer Reverse: 10 pmol, Taq Polymerase: 1 U (5 U/μL), Template DNA: 100 ng and dH_2O were used to obtain a total of 50 μL and PCR thermal cycling procedure was as follows: 95 °C 10' initiation, 95 °C 45", 59° C 30", 72° C 30" 35 cycles, 72 °C 10" termination.

Agarose gel imaging–evaluation

The resulting primers were stained with ethidium bromide on 2% agarose gels and agarose findings were evaluated by examination under ultraviolet light.

2.6. Statistical analysis

Statistical analysis was performed after evaluating the appropriateness of normal distribution. As the data were not suitable for parametric tests, intragroup comparisons were performed using Wilcoxon's test, whereas intergroup comparisons were performed by means of the Mann–Whitney U test. The Kruskal–Wallis test was applied for intergroup comparisons when more than two groups were involved.

3. Results

3.1. Audiological evaluation

In our study, we evaluated S/N - R parameters using the $2f_1 - f_2$ mode in DPOAE recordings to investigate the effect of salicylate on the motor protein prestin in electromotile responses of outer hair cells. There was no statistically significant difference in DPOAE S/N-R responses across the groups during the baseline measurements ($P > 0.05$; Figure 2). The S/N-R values in DPOAE tests at all frequency levels were over 3 dB in the control groups. The S/N-R values increased from lower frequencies to higher frequencies depending on the motility of the OHCs located on the cochlea.

3.1.1. Acute effect of salicylate on DPOAE recordings

The acute effect of salicylate administration in Group 3 (Figure 3) revealed a statistically significant decrease in DPOAE S/N-R responses (between 0.75 kHz and 8 kHz)

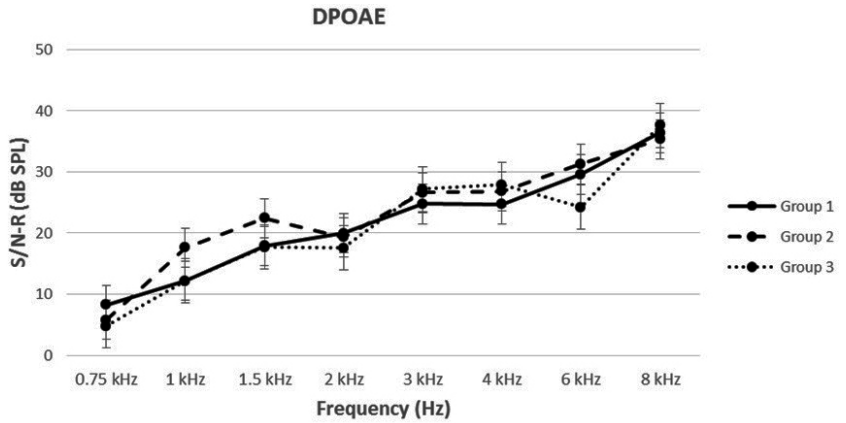

Figure 2. The mean baseline distortion product otoacoustic emission (DPOAE) signal-to-noise ratios in the study groups show that the groups were comparable.

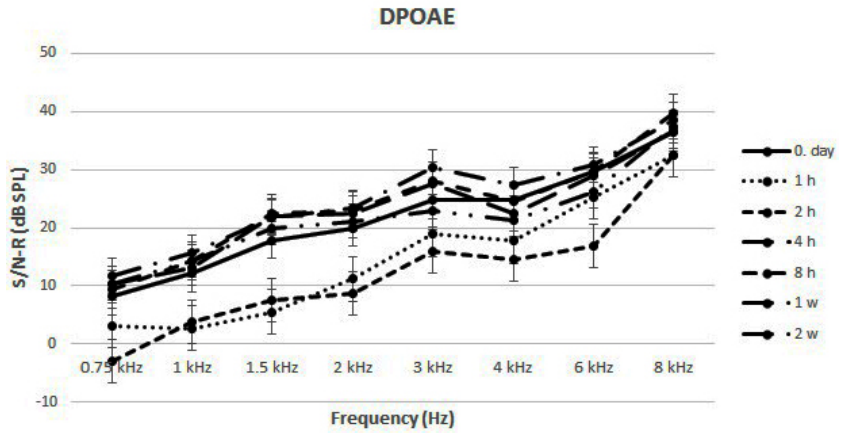

Figure 3. Distortion product otoacoustic emission (DPOAE) recordings during the whole period of sodium salicylate administration in Group 3. The figure depicts both acute (1st and 8th hours) and chronic (8th hour to 2nd week) period changes in DPOAEs.

starting from 1 h ($P < 0.01$). At 2 h after the salicylate injection, there was a statistically significant decrease in all frequencies ($P < 0.01$). In terms of the acute effect, DPOAE S/N-R responses were seen to return to baseline/normal values starting from 4 h. Evaluation of the acute effect in Group 2 (Figure 4) did not reveal a statistically significant difference among DPOAE S/N-R responses in any of the frequencies ($P > 0.05$). When the experimental group was compared against the control group at 1 and 2 h in terms of the acute effect, a statistically significant decrease was observed in all frequencies ($P < 0.01$), while there was no significant difference at 4 and 8 h ($P > 0.05$).

3.1.2. Chronic effect of salicylate on DPOAE recordings

Contrary to the acute effect, long-term salicylate administrations (Figure 3) led to a prominent increase in DPOAE S/N-R responses although the difference was not statistically significant ($P > 0.05$). While the increase was observed starting from 4 h, it was more prominent at 1 week and DPOAE S/N-R responses were observed to return to baseline/normal values at 2 weeks. Evaluation of the chronic effect with normal saline administrations (Figure 4) did not reveal a statistically significant difference among DPOAE S/N-R responses in any of the frequencies ($P > 0.05$). Consistent with the literature findings, the decreased DPOAE S/N-R responses in acute effect with increased responses and normal values in chronic effect have been interpreted as the dual effect of salicylates on outer hair cell electromotile responses. When the experimental group was compared to the control group in terms of chronic effect, there was no statistically significant difference across the frequencies ($P > 0.05$).

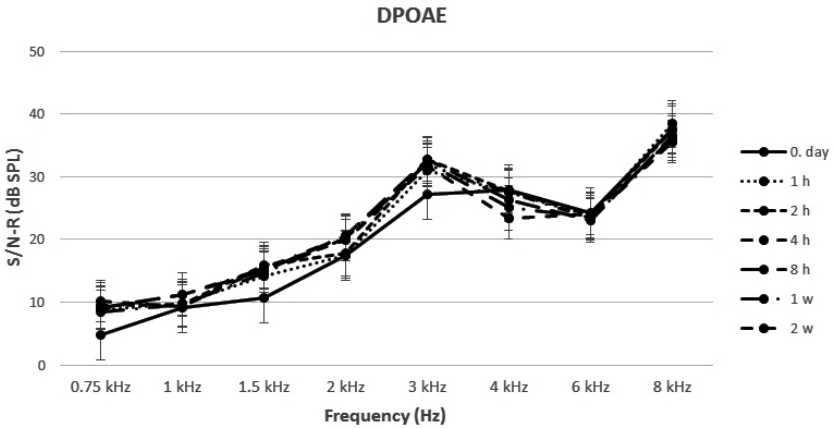

Figure 4. Distortion product otoacoustic emission (DPOAE) recordings during the whole period of saline administration in Group 2. The figure depicts both acute (1st and 8th hours) and chronic (8th hour to 2nd week) period changes in DPOAEs.

3.2. Molecular analysis

3.2.1. DNA-methylation (acute and chronic phase)

Sample DNAs were subjected to bisulfide modification to evaluate the long-term salicylate administration in prestin, the outer hair cell motor protein in guinea pig cochleae. When exon 1 of the prestin gene was evaluated with the MSP protocol, no methylation was observed in the relevant region of the prestin gene sequence on DNA in the group that was administered sodium salicylate (see Figure 5 for acute phase and Figure 6 for chronic phase). Salicylate administration for 2 h (acute) resulted in significant methylation whereas 2 weeks (chronic) administration demonstrated that the gene sequence on DNA was grossly in unmethylated form and that the relevant region was activated.

When the control groups were evaluated in terms of long-term effect at 2 weeks after the injections, both Group 1 (home-cage control) and Group 2 (placebo control) showed methylation in the prestin gene sequence region on DNA (Figure 6) and the relevant gene sequence was observed to be inactive.

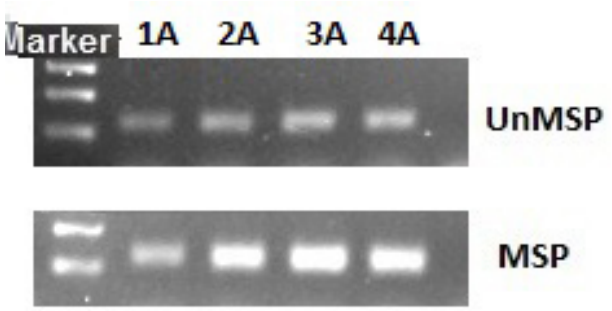

Figure 5. Polymerase chain reaction shows that sodium salicylate led to increased methylation during the first 2 h after administration (1A–4A: Group 3).

4. Discussion

The main and novel finding of this study was DNA methylation was increased during the acute phase of salicylate ototoxicity, which reversed during the chronic period. As methylation of the prestin gene blocks the expression of prestin, increased methylation during the acute phase of ototoxicity may explain the reduction in otoacoustic emission recordings during the acute phase. This study, for the first time, correlates prestin gene methylation and otoacoustic emission changes in the setting of salicylate ototoxicity.

Recent studies that have frequently investigated the influence of age, developmental factors, and environmental factors (stress etc.) on nucleotide sequences have shown gene silencing by methylation. Methylation causes only chemical changes in DNA in conditions such as carcinogenesis, while unmethylation has been associated with gene activation (20,21,27). Salicylates are well-established ototoxic agents and increased cochlear-neuronal activity has been demonstrated with chronic salicylate administration, (19,28), which often leads to reversible hearing loss and tinnitus (28,29). Studies (12,14,30) showed that tinnitus may originate from any level of the auditory system and that it is of peripheral origin if it can be acoustically masked and of central origin in the absence of such masking. In cell-based studies, according to the hypothesis of discordant damage in the outer and inner hair cell system of the cochlea, tinnitus occurs in the basilar membrane region where outer hair cells are damaged or temporarily dysfunctional while inner hair cells are preserved. This theory explains that tinnitus in patients without hearing loss is associated with diffuse damage in more than 30% of outer hair cells, which does not result in detectable hearing loss (30,31). Other studies adopt the idea that some of the adjacent nerve fibers are

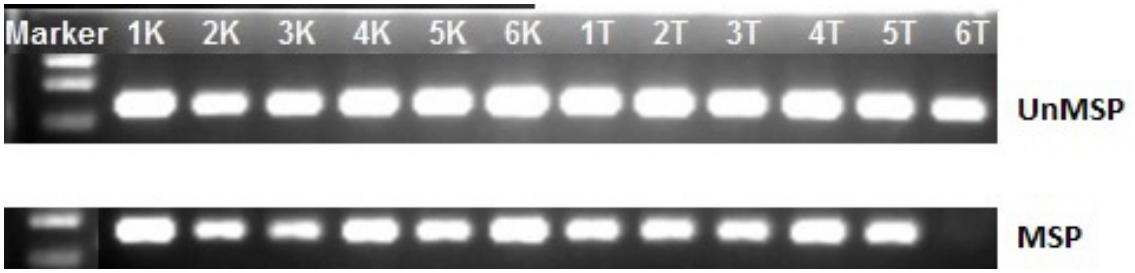

Figure 6. Results of polymerase chain reaction in all study groups at the time point of second week. (K1–K3: Group 1; K3–K6: Group 2; T1–T6: Group 3).

preserved without damage for some reason. Therefore, some artificial synapses develop between nerve fibers. These synapses between the fibers generate pathological conduction. Such conduction results in increased spontaneous activity and tinnitus (30,32). We think that tinnitus may originate from a functional disorder of lateral membrane motility of outer hair cells as the lateral membrane is in the form of a trilaminar structure, similar to a muscle triad. The motor proteins embedded in the membrane enable contractility of outer hair cells (3). The motor protein identified in recent years, namely prestin, is an extrinsic voltage sensor. It is a unit that may run independently from calcium and ATP. It changes the length of outer hair cells by using only the available Cl^- ions (3,4,7). We think that outer hair cell contractility may occur and tinnitus may develop if this motor protein loses its structure or function. Furthermore, considering the presence of three or four lines of outer hair cells, the abnormal contraction that may occur in a few cells in one line may cause tinnitus without leading to hearing loss, and it may also be a preceding indicator of an early sign for hearing loss. In recent studies, particularly in experimental tinnitus models with salicylates, decreased DPOAE levels have been observed in outer hair cells of the cochlea during the acute period, while they return to baseline levels in the chronic period (17–19). In the present study, we observed a prominent decrease in S/N-R of DPOAE measurements during the acute period (around the first and second hours), which was followed by an increase (reaching to day 0 levels) starting around the 4th hour. We think that salicylate binding to Cl^- binding domains of prestin may initially cause inactivation of outer hair cells followed by increased motile responses by replacing Cl^- ions through acute to chronic periods. Research (17) that demonstrated the increased gene expression of prestin caused increased electromotile responses in chronic period support our hypothesis. However, these molecular studies have been limited to the cochlea and the molecular factors that may have favorable or unfavorable effects on cochlea have not been comprehensively investigated.

In living species, all macromolecular structures are determined by nucleotide sequences of the genome. However, there is another mechanism that may also determine gene expression and be inherited and transmitted from one cell to another. This mechanism is referred to as the epigenetic code (27,33). DNA sequence does not change in any way during the generation of this code. DNA methylation is the most commonly seen epigenetic modification in the mammalian genome (21). DNA methylation is a chemical change of DNA that is inherited and it may be removed later in a way to obtain the initial sequence. Therefore, it belongs to the epigenetic code and is the best-characterized epigenetic mechanism (27). DNA methylation is the addition of a methyl group to DNA, e.g., addition to the 5th carbon of the pyrimidine ring in cytosine leads to decreased gene expression. DNA methylation in the C-5 position of cytosine has been observed in all vertebrates (27,33,34). The DNA methylation in adult somatic tissues typically occurs in CG dinucleotide sequences. Long-term memory processing is regulated by DNA methylation in humans. DNA methylation is necessary for normal development and is associated with imprinting, X-chromosome inactivation, inhibition of repetitive elements, and carcinogenesis (34–36). Excessive methylation (hypermethylation) occurs in CpG islands of gene promoter, leading to inhibited transcription during the course of several diseases such as cancer (37).

Investigating the epigenetic modifications on exon 1 of the prestin gene with chronic salicylate administration in our study revealed that this region of the gene was not methylated and remained unmethylated, and the fact that this gene may activate or inactivate itself through methylation when age, developmental factors, and environmental factors are involved provides some clues regarding the cochlear origin of tinnitus. We think that our findings may shed light on molecular factors that may affect prestin and eventually contribute to the development of tinnitus.

Acknowledgments

This study received financial support (TUBAP-Trakya University Unit of Scientific Research Projects-No: 2014/14).

References

1. Kachar B, Brownell R, Altschuler R, Fex J. Electrokinetic shape changes of cochlear outer hair cells. Nature 1986; 322: 365-368.
2. Ashmore JF, Mammano F. Can you still see the cochlea for the molecules? Curr Opinion Neurobiol 2001; 11: 449-454.
3. Dallos P, Falkler B. Prestin, a new type of motor protein. Nat Rev Mol Cell Biol 2002; 3: 104-111.
4. Zheng J, Shen W, He Dz, Long KB, Madison LD, Dallos P. Prestin is the motor protein of cochlear outer hair cells. Nature 2000; 405: 149-155.
5. Dallos P, Wu X, Cheatham MA, Gao J, Zheng J, Anderson CT, Jia S, Wang X, Cheng WH, Sengupta S et al. Prestin-based outer hair cell motility is necessary for mammalian cochlear amplification. Neuron 2008; 58: 333-339.
6. Liberman MC, Gao J, He DZ, Wu X, Jia S, Zuo J. Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. Nature 2002; 419: 300-304.
7. Oliver D, He DZ, Klöcker N, Ludwig J, Schulte U, Waldegger S, Ruppersberg JP, Dallos P, Fakler B. Intracellular anions as the voltage sensor of prestin, the outer hair cell motor protein. Science 2001; 292: 2340-2343.
8. Hakizimana P, Fridberger A. Effects of salicylate on sound-evoked outer hair cell stereocilia deflection. Pflugers Arch – Eur J Physiol 2015; 467: 2021-2029.
9. Tunstall MJ, Gale JE, Ashmore JF. Action of salicylate on membrane capacitance of outer hair cells from the guinea-pig cochlea. J Physiol 1995; 485: 739-752.
10. Alvan G, Berninger E, Gustafsson LL, Karlsson KK, Paintaud G, Wakelkamp M. Concentration–response relationship of hearing impairment caused by quinine and salicylate: pharmacological similarities but different molecular mechanisms. Basic & Clinical Pharmacology & Toxicology 2017; 120: 5-13.
11. Guitton MJ, Caston J, Ruel J, Johnson Rm, Pujol R, Puel JL. Salicylate induces tinnitus through activation of cochlear NMDA receptors. J Neurosci 2003; 23: 3944-3952.
12. Chan Y. Tinnitus: etiology, classification, characteristics, and treatment. Discov Med 2009; 8: 133-136.
13. Nicolas PC, Faulconhridge RL, Guitton M, Puel JC, Mondain M, Uziel A. Characteristics of tinnitus and etiology of associated hearing loss: a study of 123 patients. International Tinnitus Journal 2002; 8: 37-44.
14. Zenner HP, Vonthein R, Zenner B, Leuchtweis R, Plontke SK, Torka W, Pogge S, Birbaumer N. Standardized tinnitus-specific individual cognitive-behavioral therapy: a controlled outcome study with 286 tinnitus patients. Hear Res 2013; 298: 117-125.
15. Tonndorf J. Acute cochlear disorders: the combination of hearing loss, recruitment, poor speech discrimination and tinnitus. Ann Otol Rhinol Laryngol 1980, 89: 39-44.
16. Martin WH, Schwegler JW, Scheibelhoffer J, Ronis ML. Salicylate induced changes in cat auditory nerve activity. Laryngoscope 1993; 103: 600-604.
17. Yu N, Zhu ML, Jhonson B, Liu YP, Jones RO, Zhao HB. Prestin up-regulation in chronic salicylate (aspirin) administration: an implication of functional dependence of prestin expression. Cell Mol Life Sci 2008; 65: 2407-2418.
18. Parazzini M, Hall AJ, Lutman ME, Kapadia S. Effect of aspirin on phase gradient of 2F1–F2 distortion product otoacoustic emissions. Hear Res 2005; 205: 44-52.
19. Huang ZW, Luo Y, Wu Z, Tao Z, Jones RO, Zhao HB. Paradoxical enhancement of active cochlear mechanics in long-term administration of salicylate. J Neurophysiol 2005; 93: 2053-2061.
20. Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, Scandura JM. DNA methylation of the first exon is tightly linked to transcriptional silencing. PLoS ONE 2011; 6: 1-12.
21. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. Non-CpG methylation is prevalent in embryonic stem cells may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci USA 2000; 97: 5237-5242.
22. Jastreboff PJ, Brennan JF, Coleman JK, Sasaki CT. Phantom auditory sensation in rats: An animal model for tinnitus. Behav Neurosci 1988; 102: 811-822.
23. Jastreboff PJ, Brennan JF. Evaluating the loudness of phantom auditory perception (tinnitus) in rats. Audiology 1994; 33: 202-217.
24. Cazals Y, Horner KC, Huang ZW. Alterations in average spectrum of cochleoneural activity by long-term salicylate treatment in the guinea pig: a plausible index of tinnitus. J Neurophysiol 1998; 80: 2113-2120.
25. Le CS, Avan P, Gilain L, Romand R. CD1 hearing impaired mice. I: distortion product otoacoustic emission levels, cochlear function and morphology. Hear Res 1998; 120: 37–50.
26. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002; 18: 1427-1431.
27. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature Genetics 2003; 33: 245-254.
28. Evans EF, Borerwe TA. Ototoxic effects of salicylates on the responses of single cochlear nerve fibres and on cochlear potentials. Br J Audiol 1982; 16: 101-108.
29. Jastreboff PJ. Phantom auditory perception (tinnitus): mechanisms of generation and perception. Neurosci Res 1990; 8: 221-254.
30. Eggermont JJ. Tinnitus: neurobiological substrates. Drug Discovery Today 2005; 10: 1283-1290.
31. Jastreboff PJ, Hazell JW. A neurophysiological approach to tinnitus: clinical implications. Br J Audiol 1993; 27: 7-17.

32. Eggermont JJ, Roberts LE. Tinnitus: animal models and findings in humans. Cell Tissue Res 2015; 361: 311-336.

33. Dodge JE, Ramsahoye BH, Wo ZG, Okano M, Li E. De novo methylation of MMLV provirus in embryonic stem cells: CpG versus non-CpG methylation. Gene 2002; 289: 41-48.

34. Haines TR, Rodenhiser DI, Ainsworth PJ. Allele-specific non-CpG methylation of the Nf1 gene during early mouse development. Developmental Biology 2001; 240: 585-598.

35. Miller C, Sweatt J. Covalent modification of DNA regulates memory formation. Neuron 2007; 53: 857-869.

36. Tucker KL. Methylated cytosine and the brain: a new base for neuroscience. Neuron 2001; 30: 649-652.

37. Sun H, Li K, Shen S. A study of the role of Notch1 and JAG1 gene methylation in development of breast cancer. Med Oncol 2016; 33: 35.