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DNA methylation in hearing-related genes in non-syndromic sensorineural hearing loss patients

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Abstract

Background Our understanding of epigenetic modifications in the inner ear is very limited. Although epigenetic regulation of genes related to individual organ- and system-limited pathologies are generally expected to be tissue-specific, DNA methylation patterns in peripheral blood (PB) are found to be associated with the presence of several diseases with no typical hematological involvement. Here, we aimed to investigate whether there is a correlation between hearing-related genes' promoter region methylation in the PB samples with the presence of non-syndromic sensorineural hearing loss (NSSHL) with an aim of future utilization of DNA methylation as biomarkers in hearing loss. The study included 26 patients with NSSHL and a control group of 20 healthy individuals. CpG islands in the promoter regions of the *GJB-2*, *GJB-6*, and *SLC24A* genes were analyzed using bisulfite sequencing, and methylation percentages were analyzed.

Results Methylation levels at the 1st region of *GJB-6* and the 1st and the 4th regions of *SLC26A4* were found to differ significantly ($p=0.039$, $p=0.042$, and $p=0.029$, respectively) between the patients and the control group. There was no statistically significant difference in methylation percentages of *GJB-2* promoters. We also found that parents' consanguinity determines the methylation levels in patients' families.

Conclusions According to our knowledge, this is the first study that investigates epigenetic changes in the PB of patients with NSSHL. Despite the small sample size, our findings indicate that DNA methylation patterns in the PB could be of use for understanding epigenetic changes in the inner ear and the clinical management of NSSHL.

Keywords NSSHL, DNA methylation, Peripheral blood, Epigenetic biomarkers, Gene promoters

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Background

Hearing loss is a partial or total loss of hearing sensitivity that could impair individuals' speech, expression, and psychosocial development. Congenital prelingual hearing losses in humans occur in approximately 1 in 1000 live births [1]. 60% of congenital hearing losses are genetic in origin and divided into syndromic or non-syndromic sensorineural hearing losses (NSSHL) according to their clinical features [2]. The latter manifests with structural and functional alterations that are limited to inner ear components (especially to the cochlea) and constitute 70% of the congenital group [2]. Since the discovery of the first of its kind in 1995, 119 genes have been associated with NSSHL [3, 4].

Amongst the numerous genes that have been associated with NSSHL so far, the following three are the most commonly affected genes that have been identified in different populations around the world: Gap Junction Beta 2 (*GJB-2/CX26*), Gap Junction Beta 6 (*GJB-6*) and solute carrier family 26, member 4 (*SLC26A4*); encoding Connexin 26 (*Cx26*), Connexin 30 (*Cx30*), and Pendrin, respectively [5–8]. *Cx26* and *Cx30* are the building blocks of gap junction channels for intercellular communication of neighboring cells and are expressed in many tissues, including the inner ear. Pendrin, however, is an ion transporter and is predominantly expressed in the thyroid gland and the inner ear [9].

Although many mutations have been identified in the genes, including the ones encoding epigenetic regulators, of patients with hearing loss, our understanding of epigenetic regulation in the inner ear is considerably more limited compared to other tissues in the body, probably due to difficulties in accessing the cells of the auditory system [10]. However, epigenetic signatures in peripheral blood (PB) could potentially serve as easily accessible markers to better understand the pathophysiology of hearing and serve as tools to guide clinical decision-making.

DNA methylation is a fundamental epigenetic modification crucial for the regulation of gene expression in various organisms, including humans. This biochemical process involves the addition of a methyl group to the cytosine base within a DNA molecule, typically occurring at cytosine-guanine dinucleotide (CpG) sites. DNA methylation plays a pivotal role in controlling gene activity by influencing the accessibility of the transcriptional machinery to specific genomic regions. It is associated with diverse biological processes, such as development, cell differentiation, and maintenance of genomic stability. Developments in molecular biology technologies have let us unveil the role of epigenetic regulation in several complex diseases, such as some cancer types: colon, hepatocellular, ovarian, breast, prostate, head and neck, nasopharyngeal, esophageal, and thyroid cancer [11–13]; some neurodegenerative disorders: Alzheimer's disease, Parkinson's disease, and Huntington's disease [14]; some neuropsychiatric disorders: bipolar disorder, autism, schizophrenia, and major depression [15, 16]; and some types of inflammatory disorders: rheumatoid arthritis, knee osteoarthritis, myalgic encephalomyelitis/chronic fatigue, Crohn's disease, primary sclerosing cholangitis, and familial Mediterranean fever [17, 18]. Intriguingly, in some of these diseases with clinical manifestations limited to the neurological/neuropsychiatric spectrum, such as Parkinson's disease, Huntington's disease, and autism, epigenetic changes could be detected in the PB [14–16].

Although a relatively good wealth of information about epigenetics exists for other tissues, there is a notable lack of data about epigenetic modifications in the inner ear, a sensorineural organ [10]. To our knowledge, neither epigenetic modifications of target genes of our study (*GJB2*, *GJB6*, *SLC26A4*) nor the presence of epigenetic changes in isolated hearing losses have been investigated so far. Considering the literature that we summarized above; we aimed to investigate correlations of DNA methylation patterns in PB of isolated sensorineural hearing loss with a future prospect of utilizing these findings to develop biomarkers.

Methods

Blood sampling

Clinical investigations were performed after obtaining permission from the local ethical committee (no: 2013-7). The study was designed as a randomized controlled double-blind prospective study of 26 patients (14 male and 12 female; age between 2 and 6; mean was 4.2 ± 2.2) and 22 controls (12 male and 10 female; age between 2 and 8; mean was 4.7 ± 2.5) who were diagnosed with prelingual NSSHL and underwent cochlear implantation surgery, and who were admitted with normal hearing and lack of syndromic findings to the Otorhinolaryngology Clinics, respectively. All the procedures were done in accordance with the ethical standards of the Local Ethics Committee on human experimentation and with the Helsinki Declaration. Informed consent was obtained from the parents of all the participants. Five ml of PB collected from all the subjects were transferred into the ethylenediaminetetraacetic acid (EDTA) tubes and stored at − 20 °C freezer.

DNA isolation

DNA was isolated from blood samples using the Qiagen QIAmp DNA mini kit (Qiagen CA, USA). First, 20 µL of proteinase K and 200 µL of lysis buffer were added to 200 µL blood samples. After 10 min of incubation at 56 °C, 200 µL of ethanol was added. The mixture was later transferred into QIAmp spin columns and centrifuged

for 1 min at 8000×*g*. Centrifugation was repeated to drive the appropriate washing buffers through the column in each washing step, and the purified DNA was eluted in 100 µL of Elution Buffer provided by the kit. Concentrations of the isolated genomic DNA samples were quantified using the Nanodrop® Spectrophotometer device (MultiskanGo, Thermo Scientific, USA) as nanograms per microliter.

Bisulfite modification of DNA samples

Isolated genomic DNA samples were modified with bisulfite deamination reaction using Qiagen EpiTect Bisulfite kits (Qiagen, CA, USA) according to the manufacturer's instructions. Five hundred nanograms of DNA from each sample were mixed with 85 µL of bisulfite mix solution and 35 µL of DNA preservation buffer was added, and the total volume was adjusted to 200 µL. Samples were then incubated in the thermal cycler device (BioRad, USA) for a total of 5 h and at the following temperatures: 95 °C–5 min, 60 °C–25 min; 95 °C–5 min, 60 °C–85 min; 95 °C–5 min, 60 °C–175 min, respectively. Samples were later transferred to the Epitect spin columns, and appropriate buffers were added and centrifuged according to the manufacturer's directions. Finally, bisulfite-treated DNA samples were collected into 20 µL of elution buffers.

PyroMark polymerase chain reaction

From the bisulfite-treated samples, 179, 136, and 251 base pairs of *GJB2*, *GJB6*, and *SLC26A4* genes, respectively were amplified by PCR using Qiagen PyroMark Polymerase Chain Reaction (PCR) kit (Qiagen, CA, USA) including positive and negative controls. 12.5 µL of Pyromark PCR master mix, 2.5 µL of CoralLoad solution, 2 µL of forward and reverse primary mixture, 1 µL of bisulfite-modified DNA and nuclease-free distilled water were added to each tube to adjust the final volume of 25 µL per reaction. The thermal cycler (BioRad, USA) was set up for the initial activation step for 15 min at 95℃ and followed by 45 cycles of 94℃–30 s, 58℃–30 s, and 72℃–30 s. PCR reaction ended with a final 10 min of elongation step at 72℃.

PyroMark CpG sequencing analysis

Quality-controlled PCR products were then analyzed for methylation analysis by PyroMark *CpG* assay sequencing (Qiagen, USA). Briefly, *CpG* sequencing was completed in three steps. The first step was the immobilization of PCR products on Streptavidin-Sepharose magnetic beads according to the manufacturer's protocol. DNA fragments produced by PCR were then denatured in the second step and transferred into the PyroMark Q24 plate using the Pyromark Workstation. New plates were used for each patient to avoid contamination. In the final step, sequence primers were bound to those DNA fragments, and the sequencing was started.

The details of the protocol were as follows: in the first step, 2 µL of streptavidin-bound sepharose beads, 40 µL of binding buffer, and 28 µL of high-purity water were combined with 10 µL of PCR product in a 72-well plate and incubated in the shaker for 10 min at room temperature to allow PCR products binding to the sepharose beads. The procedure was repeated for each sample. In the second step, 2.5 µL of 3 µM sequencing primer and 22.5 µL of annealing buffer per sample were added into each well of the PyroMark Q24 plate. PyroMark Workstation was prepared using 50 ml of 70% ethanol, 40 ml of denaturation solution, 50 ml of wash buffer, 50 ml (H_2O-1), and 70 ml of high-purity water (H_2O-2). The plate was transferred from the shaker to the Workstation. All PCR products-sepharose beads were made to attach the vacuum filter by sequential holds in ethanol for 10 s, in denaturation solution for 10 s and the wash buffer for 15 s. Finally, the vacuum was rubbed for 2 min in the PyroMark Q24 plate after the pump was turned off so that PCR products in its filters would be transferred into the sequence mixture in the PyroMark Q24 plate. Proper washing of the vacuum filter was run on the Workstation to avoid contamination for the next batch of samples.

In the third step, PyroMark Q24 plate was placed into the Pyromark Sequencer and the sequencing reaction was performed using an appropriate cartridge (Method 005) containing related enzymes, substrate, and nucleotides of adenine, cytosine, guanine, and thymine, accordingly. The sequence to analyze and the sequence expected after bisulfite modification and nucleotide dispense queue to detect the methylation of promoter regions of the *GJB2*, *GJB6*, and *SLC26A4* genes are given in Table 1.

Sequences were run on the PyroMark Q24 sequencer, and the methylation analyses were done using PyroMark Q24 Analysis with version: 2.0.6. The presence of C nucleotide instead of T (at positions Y) was recorded as methylated, and the methylation values were calculated in percentages (Figs. 1 and 2).

Statistical analysis

SPSS 22.0 program was used in the statistical analysis of the data. In addition to descriptive statistical methods (mean, standard deviation), Student's *t*-test was used to compare quantitative data. Quantitative data related to each other were compared using paired *t* test. p values < 0.05 were considered as significant.

Table 1 Promoter sequences of interest for methylation analyses of GBJ2, GBJ6, and SLC26A4 genes, the expected sequence after bisulfite modification, and nucleotide dispensing queue of PyroMark Q24 sequencer

GJB 2	
Sequence to analyze	5′-GGCGCCGCTGGTGCCGGCGAAGCCCCGC-3′
Sequence expected after bisulfite modification	5′-GGYGTYGTTGGTGTYGGYGAAGTTTTYGT-3′
Nucleotide dispense queue	TGTCAGTCGTGTAGTCAGTCGATGTTCG
GJB 6	
Sequence to analyze	5′-GTTCCCTCGAGGGCCTGAGCAGGCGCCCCACACCTGCACCCGT-3′
Sequence expected after bisulfite modification	5′-GTTTTTTYGAGGGTTTGAGTAGGYGTTTTATATTTGTATTYGT-3′
Nucleotide dispense queue	TGTTCGATGTGACGTCAGTCGATTAGTATGTATCG
SLC26A4	
Sequence to analyze	5′-CGCCCGGCCCGGGCTCCACTCCCGGGGAGGCCTCGAGGGTTGCGGA-3′
Sequence expected after bisulfite modification	5′-YGTTYGGTTYGGGTTTTATTTTYGGGGAGGTTTYGAGGGTTGYGGA-3′
Nucleotide dispense queue	GTCGTCGTCGTTAGTTCGGATGTTCGAGTGTCG

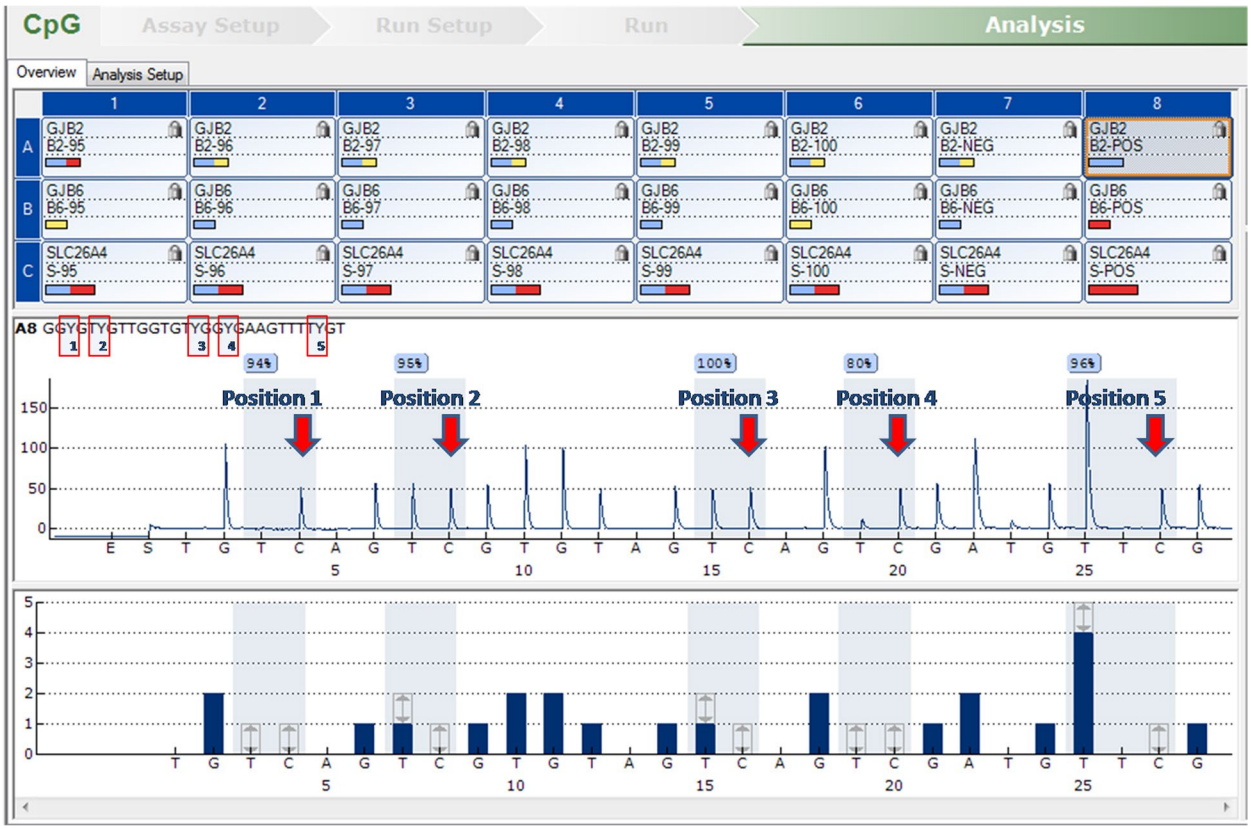

Fig. 1 Methylated CpG islands in the positive control of *GJB2* gene. Methylations were detected in all five regions as expected, which is marked by the persistence of Thymine (T) presence instead of being converted to Cytosine (C) (red arrows). The upper table shows the layout of samples in the equipment. All three genes were run together: *GBJ2* in row A, for *GJB6* in row **B** and *SLC26A4* in row **C**. Positive and negative controls for the bisulfite conversion are in columns 7 and 8. The middle section shows the sequencing results of the current run, and the bottom part shows the expected sequencing results. The small red rectangle frames show the positions where C to T substitutions occur. Nucleotides at these positions are shown in the middle graph

Results

Demographic data of the study group are summarized in Supplementary Table S1. The study group consists of 26 patients. Fourteen patients have consanguineous parents, which were distributed as the following according to their degree of kinship: four were 1st-degree, five

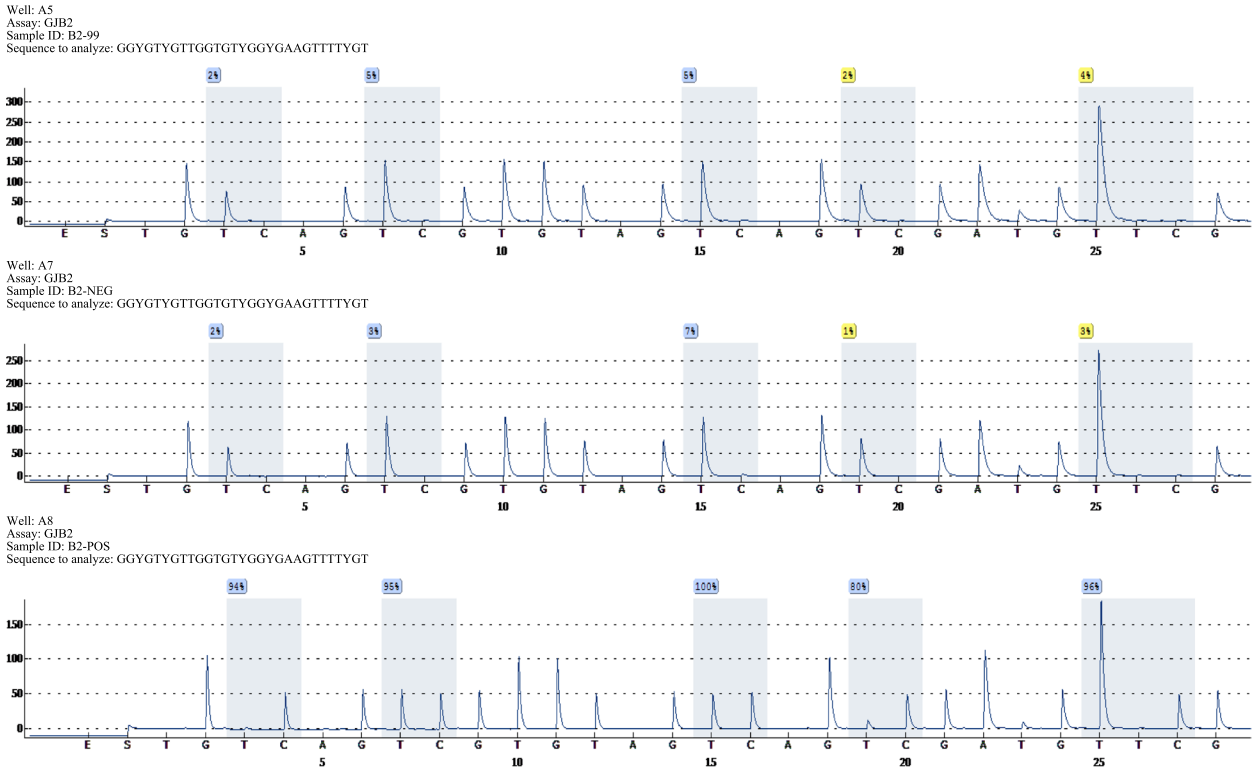

Fig. 2 Example pyrogram. Methylation percentages of *GJB2* gene for patients (first line), negative control (second line), and positive control (third line) are shown. Blue zones indicate the positions of C to T nucleotide substitution. The zones of no C to T nucleotide substitution indicate the presence of methylation as in the positive control. These percentages indicate the presence of C nucleotides, i.e., the percentages of methylation

were 2nd-, and five were 3rd-degree relatives. The control group consists of 20 individuals without hearing impairment or syndromic manifestations. We measured and compared methylation percentages at five promoter regions of *GJB2*, three promoter regions of *GJB6,* and six promoter regions of *SLC26A4* genes in the study and control groups. We also performed an analysis according to the kinship status of the study group.

The mean values and the standard deviations, *p*, and *t* values of the statistical comparison of methylation levels of the *GJB2* gene from the patient and control groups of the statistical comparison of the methylation percentages are listed in Table 2. There was no statistically significant difference in methylation percentages of the patient and control groups in all five methylation regions.

Mean values of the methylation percentages in the promoter region of the *GJB6* gene, and the statistical significance of differences are illustrated in Table 3. The difference at the first region (*GJB6.posi.1*, decreased methylation compared to the control group) was statistically significant between the patient and control groups ($p = 0.039$). The mean methylation rates of the 2nd *(GJB6.posi.2)* and the 3rd *(GJB6.posi.3)* regions in patients did not differ significantly compared to the controls ($p = 0.77$, $p = 0.104$, respectively).

The mean methylation percentages, standard deviations, and the *p* and *t* values of the promoter region of the *SLC26A4* gene are presented in Table 4. Statistically significant differences were detected in the 1st (*SLC26A4.posi.1*, decreased methylation compared to the control group) and the 4th (*SLC26A4.posi.4*, increased methylation compared to the control group) regions ($p = 0{,}042$, $p = 0{,}029$, respectively). There was no statistically significant difference in the 2nd, 3rd, 5th, and 6th regions compared to control group ($p = 0{,}858$, $p = 0{,}784$, $p = 0{,}886$, $p = 0{,}165$, respectively).

When methylation rates in the *GJB2*, *GJB6,* and *SLC26A4* genes were compared between the patients and their parents, and in between the parents by using *t* test, no statistically significant difference was observed, as similar as in between consanguineous and non-consanguineous families, in particular.

As we saw no difference between methylation levels of patients and parents but between patients and controls, we decided to investigate if the methylation levels of parents also differ from the controls. Consistent with

Table 2 The average percentages of methylation on the GJB2 gene

	Group	*n*	Mean	Std. Dvt	*p* value	*t* value
GJB2.posi.1	Patient	26	4,31	1,76	0.356	−0.933
	Control	20	4,75	1,26		
GJB2.posi.2	Patient	26	7,60	16,2	0.450	0.762
	Control	20	4,83	1,06		
GJB2.posi.3	Patient	26	7,47	10,4	0.309	1.030
	Control	20	5,05	1,21		
GJB2.posi.4	Patient	26	2,83	3,51	0.353	0.939
	Control	20	2,08	0,70		
GJB2.posi.5	Patient	26	4,63	1,33	0.606	−0.520
	Control	20	4,85	1,37		

n number, *posi* position, *Std. Dvt*. standard deviation

Table 3 The average percentages of methylation on the GJB6 gene

	Group	*n*	Mean	Std. Dvt	*p* value	*t* value
GJB6.posi.1	Patient	26	6,42	1,98	0.039*	2.138
	Control	20	5,47	0,97		
GJB6.posi.2	Patient	26	9,37	1,74	0.770	−0.294
	Control	20	9,60	3,44		
GJB6.posi.3	Patient	26	5,07	1,40	0.104	1.663
	Control	20	4,42	1,16		

n number, *posi* position, *Std. Dvt*. standard deviation

Table 4 The average percentages of methylation on the SLC16A4 gene according

	Group	*n*	Mean	Std. Dvt	*p* value	*t* value
SLC26A4.posi.1	Patient	26	5,76	1,41	0.042*	−2.089
	Control	20	6,71	1,69		
SLC26A4.posi.2	Patient	26	4,50	1,27	0.858	−0.180
	Control	20	4,56	0,81		
SLC26A4.posi.3	Patient	26	4,40	1,45	0.784	−0.276
	Control	20	4,51	0,99		
SLC26A4.posi.4	Patient	26	6,16	2,38	0.029*	2.284
	Control	20	4,99	0,97		
SLC26A4.posi.5	Patient	26	6,39	2,43	0.886	0.144
	Control	20	6,30	1,46		
SLC26A4.posi.6	Patient	26	12,03	3,44	0.165	1.414
	Control	20	10,93	1,70		

n number, *posi* position, *Std. Dvt*. standard deviation

our expectations, furthermore, we observed a significant increase in methylation levels at *GJB6.posi.1* ($p = 0.031$ for mothers, $p = 0.038$ for fathers) and *SLC26A4.posi.4* ($p = 0.012$ for mothers, $p = 0.019$ for fathers) of patients' parents, when compared to the control group. When the study group was divided according to their kinship (consanguineous vs non-consanguineous), no difference in methylation levels in the patients and their parents in consanguineous marriages was found, except for increased methylation levels at *SLC26A4.posi.4* ($p = 0.032$) of the fathers compared to the control group. However, methylation changes in families of non-consanguineous marriages are more pronounced, affecting three methylation regions. While *SLC26A4.posi.4* methylation

levels were significantly increased in patients ($p = 0.013$) and mothers ($p = 0.03$), *GJB2.posi.1* methylation levels are significantly decreased in patients ($p = 0.042$) and fathers ($p = 0.001$). *GJB2.posi.2* methylation levels were decreased; however, this observation was limited to fathers ($p = 0.008$).

Discussion

Hearing loss is one of the common sensory disorders which leads to alterations/deterioration in speech, expression, cognitive, and psychosocial development [1]. It is a multifactorial disease, that develops due to environmental (noise exposure, exposure to ototoxic drugs, viral infections, etc.), genetic factors (mutations in the nuclear or mitochondrial genes), and the combinations thereof [4, 5]. Epidemiological studies have demonstrated that one in every thousand children is born with sensorineural hearing loss [2].

While there is a significant number of studies in the literature exploring genetic foundations of hearing loss and the inner ear development, epigenetic studies of the inner ear and hearing apparatus are very limited due to the tissue-specificity of epigenetic modifications and technical barriers preventing the study of the components of hearing apparatus, particularly of the hair cells [19–21]. The most important technical limitation of animal studies is that there is a limited number of hair cells available in each specimen [22, 23]. More obstacles exist for human studies as biopsies of the inner ear are neither technically feasible nor ethically justifiable, and postmortem studies bring their own unique difficulties for study design [24]. Most of our understanding of the epigenetics of the inner ear is based on studies of genetic alterations in generic epigenetic regulators such as DNA methylases and histone deacetylases in syndromic hearing losses, cell culture studies, extrapolations from genetic studies of hearing loss and general epigenetic regulation/mechanisms, studies of epigenetic alterations in PB due to hearing losses that affect multiple organs/systems or of environmental origin [25–27]. Thus, epigenetic mechanisms in the hearing apparatus itself have remained largely unexplored. These limitations are particularly an issue for understanding epigenetic underpinnings of non-syndromic hearing loss, which exhibits clinical manifestations limited to the inner ear alone. However, growing evidence suggests that the presence of many diseases that seem to be limited to a single organ or system can correlate with epigenetic changes in the PB [11–18]. This phenomenon has been studied in neurodevelopmental disorders, which exhibit resemblance to pre-lingual hearing losses such as the affected organs sharing similar embryonic origins and presenting similar tissue accessibility issues. Therefore, we investigated if such a correlation would also apply to non-syndromic hearing losses. Our results suggest that epigenetic markers in PB correlate with the presence of non-syndromic pre-lingual hearing loss. These results are very intriguing as non-syndromic hearing loss' clinical presentation is limited to inner ear functions and goes against the existing paradigm of organ/tissue limited nature of epigenetic changes in genes expressed in a tissue-specific manner. Furthermore, none of the genes that we investigated are known to be expressed or related to any pathology in PB cells [28]. Thus, the results of this study contribute to the expanding scientific corpus that challenges our current understanding of epigenetic changes in disease. In addition to these theoretical implications, these results suggest that DNA methylation patterns in PB cells can be utilized as biomarkers to detect methylation changes in the inner ear for clinical and research purposes. Using PB methylation as a proxy for methylation in the inner ear cells can offer many insights into cellular mechanisms of congenital and acquired hearing loss, predict ototoxicity and treatment response, and transform the clinical management of otology cases.

Our results also pointed out that the disease-correlated methylation level alterations in the hearing loss-related genes that we investigated were mostly observed in the patients born from non-consanguineous marriages and their parents. These results suggest that, compared to the consanguineous counterparts, epigenetics factors might play a more prominent role in the development of NSSHL in individuals born from non-consanguineous marriages. More extensive studies in the future can shed light on the differential role of epigenetic and genetic factors in patients who were born from parents with different degrees of kinship.

Conclusions

To our knowledge, this is the first study to explore epigenetic changes in peripheric blood from patients with non-syndromic pre-lingual hearing loss, which is characterized by symptoms limited to the inner ear functions. We identified epigenetic changes in the regulatory promoter sequences at the 1st CpG region of GJB6, and in the 1st and the 4th CpG region of SLC26A4 genes from patients with pre-lingual NSSHL. These results suggest that the epigenetic changes that are thought to be limited to the inner ear might affect other unrelated tissues at a subclinical level. Another important implication of our study is that the future developments of blood DNA methylation panels can be utilized as biomarkers for clinical and research purposes in hearing loss. Finally, our results suggest that epigenetic factors might play a more important role in

NSSHL patients born from non-consanguineous parents. Further studies are needed to confirm and expand on these conclusions, including conducting studies that explore epigenetic markers for the vestibular system.

Abbreviations

NSSHL	Non-syndromic sensorineural hearing loss
GJB-2	Gap Junction Beta 2
GJB-6	Gap Junction Beta 6
SLC26A4	Solute Carrier Family 26, Member 4
Cx26	Connexin 26
Cx30	Connexin 30
PB	Peripheral blood
PCR	Polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43163-023-00555-4.

Additional file 1: Supplementary Table S1. Demographic characteristics.

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Not applicable.

Authors' contributions
OKE: conception, design of the study, collecting and analyzing the data, writing the manuscript. SC: conception, design of study, writing the manuscript. MTK: conception, design of study, writing the manuscript, collecting data, critical review. BDT: collecting data. SY: collecting and analyzing the data. ÖY: collecting data. MYD: collecting data. EA: statistical analysis. İA: collecting data, critical review. All authors read and approved the final manuscript.

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Availability of data and materials
The data supporting this study's findings are available on request from the corresponding author. The data are not publicly available due to privacy.

Declarations

Ethics approval and consent to participate
This research was conducted in the Otorhinolaryngology Clinic of Medeniyet University Goztepe Training and Research Hospital. Medeniyet University Non-Interventional Research Ethics Committee approved the study with the decision number: no: 2013-7. It was conducted in accordance with the ethical principle stated in the Declaration of Helsinki, and written consent was obtained from the parents of all the participants.

Consent for publication
The parents of the participants gave written informed consent for the publication of the data and materials contained within this study.

Competing interests
The authors declare that they have no competing interests.

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