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Lack of Methylation Changes in *GJB2* and *RB1* Non-coding Regions of Cochlear Implant Patients with Sensorineural Hearing Loss

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Abstract

Objective.—Recent advances in epigenetic studies continue to reveal novel mechanisms of gene regulation and control, however little is known on the role of epigenetics in sensorineural hearing

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Author Disclosure

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loss (SNHL) in humans. We aimed to investigate the methylation patterns of two regions, one in *RB1* and another in *GJB2* in Filipino patients with SNHL compared to hearing control individuals.

Methods.—We investigated an *RB1* promoter region that was previously identified as differentially methylated in children with SNHL and lead exposure. Additionally, we investigated a sequence in an enhancer-like region within *GJB2* that contains four CpGs in close proximity. Bisulfite conversion was performed on salivary DNA samples from 15 children with SNHL and 45 unrelated ethnically-matched individuals. We then performed methylation-specific real-time PCR analysis (qMSP) using TaqMan® probes to determine percentage methylation of the two regions.

Results.—Using qMSP, both our cases and controls had zero methylation at the targeted *GJB2* and *RB1* regions.

Conclusion.—Our study showed no changes in methylation at the selected CpG regions in *RB1* and *GJB2* in the two comparison groups with or without SNHL. This may be due to a lack of environmental exposures to these target regions. Other epigenetic marks may be present around these regions as well as those of other HL-associated genes.

Keywords

GJB2; hearing loss; methylation; qMSP; RB1; sensorineural

INTRODUCTION

An estimated 50% of hearing loss (HL) is believed to be genetic in origin, with 122 non-syndromic hearing loss genes identified to date and unique population-specific variants being continually discovered.[1] On the other hand, there is little evidence on the role of epigenetics in HL. Epigenetics includes numerous biological processes that result in a change in phenotype without altering the DNA sequence, most commonly due to histone acetylation or DNA methylation.[2] DNA methylation in promoter regions typically acts to repress gene transcription.[3] For HL, variants in the *de novo* methyltransferase *DNMT3A* affected regulation of genes involved in otic placode development, suggesting a role for DNA methylation in inner ear development.[4]

Despite advancements in investigating DNA methylation, studies on the epigenetics of HL in humans remain limited. This is mostly due to the difficulty of obtaining adequate human inner ear tissue for epigenetic study. Studies on mouse inner ear tissues are more numerous[2,3,5], for example, in a mouse model of the aging cochlea, connexin 26 expression decreased with concomitant hypermethylation of the promoter region of its encoding gene, *GJB2*[6]. Although mouse models could potentially lead to epigenetic targets in humans, a study that applied the human methylation profiling platform Illumina Infinium MethylationEPIC to mouse samples showed only 1.6% of probes to be conserved[7], indicating low correspondence between human and mouse methylomes. Previously in Chinese children with sensorineural hearing loss (SNHL), exposure to lead and cadmium was associated with differentially-methylated regions in the *RB1* promoter.[8] Currently, this is the only study that utilized human subjects for an epigenetic study on SNHL, with blood utilized as a surrogate tissue for the inner ear. Recent evidence suggests that salivary DNA is a better surrogate than blood for brain and neural tissue for methylation studies[9,10] and

possibly the neuroepithelium of the inner ear, which would allow direct investigation of DNA methylation in humans without performing invasive sampling. In this study, we aimed to investigate DNA methylation patterns in non-coding regions of *GJB2* and *RB1* using salivary DNA of Filipino patients with SNHL.

MATERIALS AND METHODS

Ethical approval was obtained from the University of the Philippines Manila Research Ethics Board and the institutional review board of the Baylor College of Medicine and affiliated hospitals. Informed consent was obtained from all study participants.

Salivary DNA samples from 15 Filipino patients who underwent cochlear implantation (CI) for SNHL (ages 3.5–21 years, 67% female; Table 1) and 45 unrelated Filipinos were utilized in this study. CI patient DNA was previously used in investigations on damaging variants in hearing loss, otitis media, and temporal bone anomalies.[11–15] Control DNA samples were previously isolated from saliva of a cohort of Filipino-descent individuals who did not have hearing loss and were recruited for a study on speech delay.[16] Bisulfite conversion of DNA samples was performed as described in the EpiTect Bisulfite Kit (Qiagen).

Two CpG-rich regions were investigated. The *RB1* promoter sequence contains five CpG sites (hg19 chr13:48877688, chr13:48877674, chr13:48877677, chr13:48877679, chr13:48877684).[17] Additionally, we investigated a region located in an enhancer-like region (EH38E1658502) between exons 1 and 2 of the SNHL-associated gene *GJB2*. This region contains four CpGs in close proximity (hg19 chr13:20766381, chr13:20766387, chr13:20766397, chr13:20766399) as shown in iMETHYL and the ENCODE project.[18,19] For the *GJB2* region, primers for methylation-specific qPCR (qMSP) were designed using MethPrimer software.[20] qMSP using TaqMan® probes was then performed using the EpiTect Methylight PCR kit (Qiagen) using gene-specific primer and probe sets (Table 2).

Standardization was done using the EpiTect PCR Control DNA Set (Qiagen) which contains 100% methylated and 100% unmethylated control DNA. Additionally, 75%, 50%, and 25% methylated controls were prepared. Percentage methylation was calculated using the following formula:

$$C_{meth} = 100/[1 + 2^{(CT_{FAM}-CT_{VIC})}]\%$$

where C_{meth} is percentage methylation, CT_{FAM} is threshold cycle of the methylated reporter (FAM channel), and CT_{VIC} is the threshold cycle of the unmethylated reporter (VIC channel).[21]

RESULTS

Bisulfite-converted DNA samples were of high concentration and purity based on nanodrop measurements, 260/280 ratio and 260/230 ratio. PCR using *RB1* and *GJB2* primers produced appropriately-sized bands.

For both *RB1* and *GJB2,* the 100% methylated and 100% unmethylated BSC standards produced only FAM and VIC signals, respectively, while the 75%, 50%, and 25% methylated standards produced both FAM and VIC signals appropriately, indicating that the system was able to distinguish the methylation levels of both target regions. qMSP of bisulfite-converted salivary DNA revealed that for both CI patients and ethnically-matched controls, only VIC signals were produced. This indicates zero methylation at the targeted *RB1* promoter and *GJB2* enhancer-like regions for both cases and controls.

DISCUSSION

qMSP of bisulfite-converted salivary DNA of Filipino CI patients and non-hearing-impaired controls revealed 0% methylation at a region in the *RB1* promoter previously described as being differentially methylated in SNHL patients exposed to lead and cadmium[8] and a target sequence in an enhancer-like region in the SNHL-associated gene *GJB2*. This finding may be due to the lack of sufficient levels of environmental exposures causing differences in the target regions. Population-specific differences may also be present, as the cohort from the study by Xu et al.[8] were of Chinese descent.

Alternatively, there may be other genetic factors that could have explained this finding. It must be noted that the DNA samples from our CI patients were submitted for exome sequencing,[11–15] and in six out of fifteen patients, known variants in HL genes were not identified. Further exome analysis of these patients, however, revealed potential candidate genes for HL and temporal bone anomalies in three patients as well as novel variants in known HL genes in four patients (patients 10–15; Table 1).[15] In the other 10 patients, rare damaging variants were identified in non-*GJB2* HL genes (patients 1–9; Table 1). For this study, we hypothesized that differentially-methylated regions (DMRs) in non-coding sequences of *GJB2* or other known HL-associated genes may be present in our SNHL patients. In this study, *GJB2* was selected for methylation studies due to it being the most common HL-associated gene in many world populations[22], however we found zero methylation at the selected enhancer-like region of *GJB2*. In addition to the CpG sites we tested, several enhancer-like regions were located between the first and second exons of *GJB2*. Changes in non-coding regions in the form of epigenetic marks may be present in these *GJB2* regions, or in non-coding regions of other genes for HL.

qMSP has been criticized as being more challenging in terms of designing primers specific for methylated and nonmethylated regions[23], however, improvements in the design of protocols and kits which utilize only one primer set alongside methylation-specific TaqMan® probes as in the case of this study has allowed easier, high-throughput, and sensitive evaluation of percentage methylation of target sequences[21]. However, we were somewhat limited as to which region as well as sequence length to investigate due to the use of TaqMan® probes. A more comprehensive investigation into these CpG-rich regions, for example using pyrosequencing or amplicon sequencing following bisulfite conversion to investigate the entire stretch between exons 1 and 2 of *GJB2*, may yield DMRs associated with HL.

Other limitations of this study must be considered. First, we did not have environmental data such as chemical exposure from our cases and controls. Second, we only investigated for methylation patterns in this study, and no other means of epigenetic control. However, exome data from our CI cohort were negative for variants in microRNA genes previously associated with HL in Spanish families.[24] Third, the relatively small sample size of our cohort may not have been enough to detect any differences in methylation patterns between cases and controls. Finally, due to the aforementioned difficulty of obtaining human inner ear tissue, we utilized salivary DNA as a surrogate for inner ear neuroepithelium.

CONCLUSION

In summary, we showed that in Filipino CI patients and hearing controls, there were no changes in terms of methylation in a sequence previously described as a DMR in hearing-impaired children exposed to lead and cadmium, as well as a CpG-rich sequence in an enhancer-like region of *GJB2*. Further study into these regions as well as noncoding regions in other HL-associated genes may yield DMRs associated with HL, preferably using genome-wide methylation arrays.

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Table 1.

Clinical Descriptions and Hearing Loss (HL) Genes with Variants in Cochlear Implant Patients[11–15]

Patient No.	Prenatal History	Other Childhood Disease	Temporal Bone Findings	HL gene with variant
1	Maternal joint pain during pregnancy	Unremarkable	Superior semicircular canal dehiscence and otitis media, left	*KCNQ4*
2	Unremarkable	Unremarkable	EVA, bilateral	*SLC26A4*
3	Maternal fever at 6 mos AOG	Microscopic hematuria; primary complex	Normal	*CDH23*
4	Unremarkable	Delayed motor development; white matter disease by MRI	Normal	*WFS1*
5	Unremarkable	Primary Koch infection	Normal	*MYO15A*
6	Maternal diabetes at 6 mos AOG	Unremarkable	Malformed cochlea, bilateral; absent cochlear and inferior vestibular nerves, right	*SLC9A3R1*
7	Unremarkable	Unremarkable	EVA, bilateral	*COL4A3*
8	Unremarkable	Unremarkable	EVA, bilateral	*SLC26A4*
9	Unremarkable	Global developmental delay; left foot inversion	Normal	*MYH14*
10	Unremarkable	Maternal urinary tract infection and eclampsia during pregnancy	EVA, left	*IST1* [a]
11	Unremarkable	Global developmental delay	Normal	*SLC12A2* [b]
12	Unremarkable	Sepsis at 19 days of age and was prescribed various antibiotics, one of which was Amikacin	Normal	*MYO7A* [b]
13	Unremarkable	Turbinate hypertrophy secondary to papillary allergic rhinitis with nodule in nasopharynx	Normal	*CLDN9* [b]
14	Unremarkable	Unremarkable	Normal	*GREB1L* [b] ; *CBLN3* [a]
15	Unremarkable	Unremarkable	EVA; Otitis media, left	*GDPD5* [a]

[a] variant is found in candidate gene[15];

[b] variant is novel and found in a known HL gene[15]

Abbreviations: AOG, age of gestation; EVA, enlarged vestibular aqueduct

Table 2.

Primer and Probe Sets for RB1 and GJB2 Target Regions

	Forward primer	Reverse primer	Methylated probe	Unmethylated probe
RB1	5’-GTTTAAGGAGGGAGAGTGG-3’	5’-AAATAACTATAAA CCTCATCCCTATCC-3’	FAM5’-AACACGTCCGAACCGCGCCGAATAC3’-TAMRA	VIC5’-AACACATCCAAACCACACCAAATAC3’-TAMRA
GJB2	5’-GGAATTGATTTTTATTTTTTGGAG-3’	5’-AAAAAAACCACTAAAATCTTAACCC-3’	FAM-5’CCGAAATCGACTAACTCCGCGTTA3’-TAMRA	VIC-5’CCAAAATCAACTAACTCCACATTA3’-TAMRA

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