Association between Genetic Variations in \textit{GRHL2} and Noise-induced Hearing Loss in Chinese High Intensity Noise Exposed Workers: A Case-control Analysis

Xin LI$^{1,2}$, Xinying HUO$^{1,2}$†, Kai LIU$^{3}$†, Xiuting LI$^{1,4}$, Meilin WANG$^{1,2}$, Haiyan CHU$^{1,2}$, Feifei HU$^{1,2}$, Huanxi SHENG$^{1,4}$, Zhengdong ZHANG$^{1,2}$* and Baoli ZHU$^{1,4}$*

$^1$Department of Environmental Genomics, Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Cancer Center, Nanjing Medical University, China
$^2$Department of Genetic Toxicology, The Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, China
$^3$Department of Disease Prevention, The Third Affiliated Hospital of Nanjing Medical University, China
$^4$Institute of Occupational Disease Prevention, Jiangsu Provincial Center for Disease Prevention and Control, China

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Abstract: The grainyhead like 2 (GRHL2) is a transcription factor, and the role among noise exposed workers is not well established. We tested whether \textit{GRHL2} polymorphisms are associated with the risk of noise-induced hearing loss (NIHL) in Chinese high intensity noise exposed workers. We genotyped six polymorphisms of \textit{GRHL2} gene (i.e., rs611419, rs3779617, rs3735713, rs3735714, rs3735715, and rs6989650) of 340 NIHL cases and 356 control subjects who exposed to noise higher than 85 dB (A) [Lex, 8 h=time-weighted average of levels of noise exposure (Lex) for a nominal 8 h working day] in a Chinese population. Compared with rs611419 AA genotype, the AT/TT genotypes conferred protection against NIHL [adjusted odds ratio (OR)=0.71, 95% confidence interval (CI)=0.52–0.98]. No altered NIHL risk was associated with the other five polymorphisms. In the combined analyses, we found that the combined genotypes with three to eight variant alleles were associated with a decrease risk of NIHL compared with those with zero to two variant alleles, and the decrease risk was more pronounced among subgroups of exposure time>20 yr (0.31, 0.16–0.62) and drinkers (0.51, 0.29–0.90). Polymorphisms of \textit{GRHL2} may positively contribute to the etiology of NIHL.

Key words: Grainyhead like 2, Single nucleotide polymorphism, Association study, Molecular epidemiology, Noise-induced hearing loss

Introduction

Noise-induced hearing loss (NIHL) is the leading occupational disease and the second most frequent form of sensorineural hearing loss after age-related hearing

*To whom correspondence should be addressed.
†These authors contributed equally to this work.
E-mail: drzdzhang@gmail.com, E-mail: zhubl@jscdc.cn
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POLYMORPHISMS OF GRHL2 AND NOISE-INDUCED HEARING LOSS

impairment (ARHI). It has been an increasing problem in countries with rapidly growing industrial activity such as China. Although the exact causes of NIHL have not been identified yet, accumulating epidemiological evidence indicates that noise, chemicals like organic solvents, heavy metals, smoking, high blood pressure and cholesterol levels are associated with NIHL risk\(^1\)\(^{-4}\). However, when exposed to the same noise, some people developed NIHL while the others did not, suggesting that NIHL is a complex disease caused by a gene-environment interaction\(^3\).

Little is known about the genetic factors that may influence NIHL. However, it was demonstrated that genetic factors contribute to the susceptibility to NIHL, deduced from studies using animals\(^5\). For example, many heterozygote and homozygote knockout mice studies identified that the gene coding for otocadherin 23 (cdh23)\(^6\), plasma membrane Ca\(^{2+}\)-ATPase isofrom 2 gene (pmca2)\(^7\), glutamate peroxidase 1 (gpx1)\(^8\), and heat shock factor (hsf1)\(^9\),\(^10\) might be involved in the susceptibility of NIHL. In humans, no formal heritability studies for NIHL have been realized up to now. Only a few association studies have been performed\(^11\)\(^{-14}\).

The grainyhead like 2 (GRHL2), also known as BOM (brother of mammalian grainyhead) and TFCP2L3 (transcription factor cellular promoter 2-like 3), is a transcription factor that expressed in epithelial tissues, not only plays a central role in embryonic development, but also functions in epithelial cell maintenance throughout life\(^15\). The underlying pathological reason of GRHL2’s involvement in hearing loss such as ARHI is an impaired maintenance\(^16\). In Drosophila, grainyhead plays a critical role in the regulation of many important developmental processes, and the homozygous mutations in grainyhead are embryonic lethal\(^17\). Grainyhead-like family members (GRHL1-GRHL3) can regulate epithelial adhesion\(^18\).

The human GRHL2 gene is located on chromosome 8q22.3 and consists of 16 exons and 15 introns. Genetic polymorphisms in GRHL2 may influence the susceptibility to different types of hearing loss, i.e., progressive autosomal dominant hearing loss (DFNA28) and ARHI\(^16\),\(^18\). As to NIHL, however, to the best of our knowledge, no published study has investigated the role of GRHL2 variations in the etiology of NIHL in a Chinese population. ISO 1999 describes the harmfulness of noise whose sound pressure level is higher than 85 dB (A)\(^19\). Given the important role of GRHL2 in epithelial cell maintenance, and the association between GRHL2 polymorphisms and other hearing loss risks suggested by genetic epidemiological studies, it is conceivable that genetic variants of GRHL2 may have an effect on the developing of NIHL when workers exposed to high intensity noise. To test this hypothesis, we genotyped six GRHL2 potentially functional polymorphisms (i.e., rs611419, rs3779617, rs3735713, rs3735714, rs3735715, and rs6989650) and evaluated the associations between these six SNPs and NIHL risk in our on-going case-control study in a Chinese high intensity noise exposed population.

**Subjects and Methods**

*Study subjects and environmental noise monitoring*

The research protocol was approved by the institutional review board of Nanjing Medical University and informed consent was obtained from all study participants. All subjects were genetically unrelated ethnic Han Chinese. A total of 2,904 workers were selected. The volunteers were foundryman recruited from a machinery manufacturing corporation (Nanjing, China) and spinning workers recruited from a chemical fiber company (Yizheng, China) between April 2010 and May 2011. These regions were selected because of the high stability of the workforce and the working environment of these factories was similar while workers were all exposed to steady noise during working time. We considered that investigating the subjects who exposed to noise lower than 85 dB (A) may lead to a spurious result. Thus, in this study, the NIHL cases and control subjects were selected from these regions according to the following criteria: the subjects were recruited only among the workers exposed to noise higher than 85 dB (A); those with a history of otological disease, head injury, other diseases that could affect hearing, previous or present treatment with ototoxic drugs, and/or potentially harmful noise exposure during military service, and/or exposed to chemical or physical factors during working time (e.g., heat, vibrations) were excluded from the present study. Before recruitment, a questionnaire was administered through face-to-face interviews by trained interviewers to obtain information on demographic data, working and military history, physical and chemical exposure, previous and present medical conditions, smoking, drinking status, pharmaceutical preparations, hereditary factors, hearing protection and noise exposure at previous work places or during military service. 2,605 workers finished their interviews and the response rate was 89.7%. So, we chose 340 NIHL cases. After that, we chose 356 control subjects who were matched with these 340 cases by age, sex, exposure level and exposure time. In this study the subjects who had smoked 100 cigarettes or more
in their lifetimes were defined as smokers and the rest of them were defined as non-smokers. The subjects who had three or more alcohol drinks per week for at least one year were defined as drinkers and the others were defined as non-drinkers. Each subject donated 5 ml venous blood samples to be used for genomic DNA extraction after written informed consent was obtained.

According to the Chinese National Criteria for Noise in the Workplace (GBZ43-2002, http://www.zybwn.net), noise exposure levels were assessed with sound pressure individual noise meters (Noise-Pro, Quest, USA) which worn by 1 to 10 noise exposed workers during their working time a day, three times a year of each workplace. In the mean while, a sound pressure noise meter (Noise-Pro, Quest, USA) were used at 10 AM, 3 PM, 5 PM for three consecutive days of each workplace to test it. To evaluate the actual noise exposure level, the result was recorded by Lex, 8 h (normalization of equivalent continuous A-weighted sound pressure to a nominal 8 h a day). The noise level for each subject was steady. In Table 1, the representative value of noise exposure level which used as a demographic data for each case or control is an average of the measured values for each subject.

### Audiological assessment and definition of NIHL

Pure-tone audiometry was performed by a trained technician for both ears at 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 kHz in a sound-attenuating booth. Hearing loss can either be in the high-frequency range (3.0–6.0 kHz) or low-frequency range (0.5–2.0 kHz). We use pure-tone audiograms to distinguish NIHL: a typical pure-tone audiogram of NIHL showed a notch around 3 to 6 kHz while threshold values in the high-frequency range were substantially worse compared with low-frequency range. Hearing threshold worse than 25 dB in low frequency or/and high frequency was defined as NIHL. But all the workers with low-frequency hearing threshold values worse than 25 dB must be transferred from noise-exposed environment immediately according to Diagnostic Criteria of Occupational Noise-induced Hearing Loss. So in the present study subjects whose hearing threshold worse than 25 dB in high frequency was defined as NIHL cases, and the threshold levels were measured in the high frequency (3.0–6.0 kHz). We calculated the average of the threshold levels measured in the high frequency (3, 4 and 6 kHz) for both ears with adjustment for age and sex to represent the threshold for all the subjects.

### SNP Selection and genotyping

Recently, many genome-wide association studies (GWASs) have found that there might be some other functional polymorphisms located downstream or upstream

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### Table 1. Distribution of selected variables between the NIHL cases and controls

| Variables                  | Cases (n=340) | Controls (n=356) | p a  |
|----------------------------|--------------|-----------------|------|
|                           | n            | %               | n    | %   |      |
| Age (years) (mean ± SD)    | 39.3 ± 5.8   | 39.8 ± 5.8      | 0.217|
| <40                       | 172          | 50.6            | 165  | 46.4| 0.263|
| ≥40                       | 168          | 49.4            | 191  | 53.7|      |
| Sex                       |              |                 |      |     |      |
| Male                      | 306          | 90.0            | 317  | 89.0| 0.681|
| Female                    | 34           | 10.0            | 39   | 11.0|      |
| Exposure level [dB (A)]   | 92.9 ± 4.0   | 92.8 ± 3.4      | 0.873|
| Exposure time (years)     | 17.0 ± 6.9   | 17.0 ± 7.0      | 0.891|
| ≤20                       | 235          | 69.1            | 248  | 69.7| 0.876|
| >20                       | 105          | 30.9            | 108  | 30.3|      |
| Threshold (dB)            | 37.6 ± 11.7  | 14.2 ± 3.9      | <0.001|
| Smoking status            |              |                 |      |     |      |
| Non-smokers               | 144          | 42.4            | 152  | 42.7| 0.927|
| Smokers                   | 196          | 57.6            | 204  | 57.3|      |
| Drinking status           |              |                 |      |     |      |
| Non-drinkers              | 186          | 54.7            | 198  | 55.6| 0.809|
| Drinkers                  | 154          | 45.3            | 158  | 44.4|      |

aStudent’s t-test for age, exposure level, exposure time and threshold distributions between cases and controls; two-sided χ² test for the other selected variables between cases and controls.
of the genes, even in intergenic regions known as gene deserts. But from a gene perspective, the 5′ near gene (as promoter region), 5′ untranslated region (5′ UTR), 3′ UTR, or coding regions with amino acid changes are the most popular potentially functional regions in current association studies. So we used the SNP selection strategy which only chose the above regions. SNPs were selected from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) with a minor allele frequency (MAF) > 0.10 in Han Chinese. Potentially functional polymorphisms were identified to meet the following criteria: located in the 5′ near gene, 5′ UTR, 3′ UTR, or coding regions with amino acid changes. According to the criteria, nine SNPs were identified, seven of which were located in 3′ UTR. We then calculated correlation coefficient (R²) for each pair of the seven SNPs in 3′ UTR and found that rs3824090, rs3824091 and rs6989050 were in complete linkage disequilibrium (LD) (R²=1.00), while rs3735715 and rs3735717 were in LD too (Fig. 1). Thus the selection of rs6989650 and rs3735715 was enough, so we finally chose six SNPs in the GRHL2 gene (i.e., rs611419, rs3779617, rs3735713, rs3735714, rs3735715, and rs6989650) (Fig. 2). Total genomic DNA was extracted following standard procedures using Tiangen DNA extraction kit (Beijing, China). Genotyping was performed with the TaqMan SNP Genotyping Assay using the 384-well ABI 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Four blank controls were arranged in each plate to ensure accuracy of the genotyping. After the completion of the amplification, SDS 2.3 automated software was used for allelic discrimination. The analysis was performed by two persons in a blind fashion. More than 10% of the samples were randomly selected for repeat assays, and the results were 100% concordant.

Statistical analyses

Hardy-Weinberg equilibrium (HWE) for genotypes was tested by a goodness-of-fit χ²-test. Demographic and genotype information for NIHL cases and controls were compared using the Student’s t-test (for continuous variables) or χ²-test (for categorical variables). The associations between the genotypes of the six polymorphisms and risk of NIHL were estimated by computing odds ratio (ORs) and 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for age, gender, exposure time, and exposure level. The computation of linkage disequilibrium between polymorphisms was estimated using D’ and R², and the characterization of these patterns was showed by Haploview 4.1 software. All tests were two-sided, and p<0.05 was considered statistically significant by using SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC, USA).

Results

Characteristics of the study subjects

The frequency distributions of selected characteristics of the cases and controls are presented in Table 1. The cases and controls appeared to be well matched by age, sex, exposure level and exposure time (p=0.217, 0.681, 0.873, and 0.891 for age, sex, exposure level, and exposure time,
respectively). These variables were further adjusted in the unconditional logistic regression analysis. In addition, there was no significant difference between the cases and controls in smoking and drinking statues ($p=0.927$ and 0.809, respectively). As expected, the threshold value of NIHL cases was significant higher than the control subjects ($p<0.001$).

**Association between GRHL2 polymorphisms and risk of NIHL**

Basic information of the six SNPs in GRHL2 gene is shown in Table 2. All observed genotype frequencies in controls conformed to HWE ($p=0.613$, 0.316, 0.806, 0.883, 0.153, and 0.652 for rs611419, rs3779617, rs3735713, rs3735714, rs3735715, and rs6989650, respectively). Allele frequencies and genotype distributions of the six GRHL2 polymorphisms are shown in Table 3. For SNP rs611419, the frequencies of the AA, AT and TT genotypes were 34.8%, 45.4% and 19.8%, respectively, among the cases; and they were 27.5%, 48.6% and 23.9%, respectively, among the controls ($P_{\text{trend}}=0.038$). Furthermore, in a dominant model, rs611419 AT/TT genotypes showed more resistant to NIHL, compared with rs611419 AA genotype (adjusted OR=0.71, 95% CI=0.52–0.98). The rs611419 T allele frequency was 42.5% among the cases and 48.2% among the controls, and the difference was statistically significant ($p=0.033$). However, no significant frequency differences in genotype of the other five SNPs were observed between NIHL cases and controls ($p=0.683$, 0.588, 0.720, 0.345, and 0.737 for rs3779617, rs3735713, rs3735714, rs3735715, and rs6989650, respectively).

**Stratification analysis of the combined genotypes of the GRHL2 polymorphisms and risk of NIHL**

We then evaluated the effect of the combined genotypes of the six SNPs on NIHL risk stratified by age, gender, smoking status, drinking status and exposure time. As shown in Table 5, the subjects carrying 3–8 variant alleles showed more resistant to NIHL among workers who exposed to high intensity noise for more than 20 years (adjusted OR=0.31, 95% CI=0.16–0.62) and drinkers (0.51, 0.29–0.90), compared with subjects carrying 0–2 variant alleles.

**Discussion**

Our study aimed to evaluate the contribution of the GRHL2 potentially functional polymorphisms to NIHL susceptibility in a Chinese high intensity noise exposed population based on a case-control analysis. When evaluated separately, we observed that rs611419 AT/TT genotypes were more resistant to NIHL compared with the AA genotype. However the other five polymorphisms did not show any effect on the risk of NIHL. In the mean time, when we analyzed the effects of those six GRHL2 SNPs together, there was a significant association between the combined genotypes and NIHL ($p=0.029$). Compared with subjects carrying 0–2 variant alleles, significantly protection effect was observed in subjects carrying 3–8 variant alleles ($p=0.034$, adjusted OR=0.67, 95% CI=0.47–0.97).

**Combined analysis between the six SNPs and NIHL risk**

To evaluate whether there exists an interaction between these polymorphisms, we combined the six polymorphisms for the analysis. As shown in Table 4, there was a significant association between the combined genotypes and risk of NIHL ($p=0.029$). Compared with subjects carrying 0–2 variant alleles, significantly protection effect was observed in subjects carrying 3–8 variant alleles ($p=0.034$, adjusted OR=0.67, 95% CI=0.47–0.97).
susceptibility to NIHL in the Chinese high intensity noise exposed population. To the best of our knowledge, this is the first report to evaluate the association between $GRHL2$ polymorphisms with NIHL risk in a Chinese population. NIHL is a complex disease caused by an interaction between genetic and environmental factors. Noise itself
is absolutely the most frequent cause of NIHL. Different noise exposure results in different reaction to people. For example, at 80 dB (A) there is no significant risk in the majority of individuals. But when the noise reaches 85 dB (A) there starts a significant risk with susceptible individuals causing a significant hearing loss from a lifetime of exposure. At 90 dB (A) or above the risk becomes material, with the majority of individuals accruing a significant hearing loss. So from a preventive point of view, as long as daily noise exposures do not exceed 85 dB (A), the risk

| Combined genotypes | Cases (n=337) | p<sup>b</sup> | Adjusted OR (95%CI)<sup>c</sup> |
|--------------------|---------------|---------------|-------------------------------|
| 0–2                | 86            | 0.034         | 1.00 (reference)              |
| 3–8                | 251           | 0.67 (0.47–0.97) |

<sup>a</sup>The 0–8 represents the numbers of variants within the combined genotypes; the variant alleles used for the calculation were rs611419T, rs3779617A, rs3735713A, rs3735714T, rs3735715A, and rs6989650T; 0–2=0–2 variant alleles. <sup>b</sup>Two-sided χ² test for the distributions of genotype frequencies. <sup>c</sup>Adjusted for age, sex, exposure level and exposure time in logistic regression model.

| Variables | Cases/controls | Combined genotypes (cases/controls) | p<sup>a</sup> | Adjusted OR (95%CI)<sup>b</sup> |
|-----------|---------------|------------------------------------|---------------|-------------------------------|
| Age       |               |                                    |               |                               |
| <40       | 233/248       | 51/52                              | 0.806         | 0.93 (0.60–1.44)              |
| ≥40       | 104/108       | 35/15                              | 0.001         | 0.31 (0.16–0.62)              |
| Gender    |               |                                    |               |                               |
| Male      | 303/317       | 73/58                              | 0.077         | 0.71 (0.48–1.04)              |
| Female    | 34/39         | 41/53                              | 0.159         | 0.55 (0.19–1.62)              |
| Exposure time (years) |   |                                    |               |                               |
| ≤20       | 233/248       | 51/52                              | 0.806         | 0.93 (0.60–1.44)              |
| >20       | 104/108       | 35/15                              | 0.001         | 0.31 (0.16–0.62)              |
| Smoking status |           |                                    |               |                               |
| Non-smokers | 143/152     | 35/25                              | 0.087         | 0.60 (0.34–1.08)              |
| Smokers    | 194/204       | 51/42                              | 0.179         | 0.72 (0.45–1.15)              |
| Drinking status |           |                                    |               |                               |
| Non-drinkers | 184/198     | 47/44                              | 0.446         | 0.84 (0.52–1.35)              |
| Drinkers   | 153/158       | 39/23                              | 0.016         | 0.51 (0.29–0.90)              |

<sup>a</sup>Two-sided χ² test for the distributions of genotype frequencies. <sup>b</sup>Adjusted for age, sex, exposure level and exposure time in logistic regression model.
of hearing loss is minimal. That is why the noise at work regulation requires the employer to perform a survey if the noise level may be above 85 dB (A)\(^\text{21}\). Considering the fact that noise is harmful starting from 85 dB (A), we thought the noise lower than 85 dB (A) was not strong enough to cause hearing impairment. That is why in our study, we only investigated participants who exposed to noise above 85 dB (A).

Except for one variation of GRHL2, no statistical difference between cases and control subjects has been obtained for the analyzed SNPs. This result was similar to a previous study, also failed to identify polymorphisms in GRHL2 as NIHL susceptibility variations in a candidate gene association study in both Swedish and Polish population\(^\text{23}\). This might either indicates that the previously investigated SNPs and the five polymorphisms of this study that yielded insignificant results are not involved in NIHL at all, or that the effect of the variations on the development of NIHL are too small to be detectable with the current sample size. Alternatively, it might be that the involvement of these variations in the development of NIHL only becomes clear when the statistical analysis allows for interactions between several polymorphisms\(^\text{22}\). So when we analyzed the six polymorphisms together, we found significant difference between the combined genes and risk of NIHL. And the decrease risk of NIHL of participants with 3–8 variant alleles was particularly among subgroups of long time exposed workers and drinkers. It suggested that workers exposed to noise more than 20 years and drinkers showed more resistant to NIHL among the subjects carrying 0–2 variant alleles of the six polymorphisms. These findings indicated that there might be different mechanisms underlying the long time exposure to noise and drinking status, and the GRHL2 might affect these mechanisms differently.

Significant differences between cases and control subjects have been obtained for rs611419, indicating that GRHL2 might be regarded as a NIHL susceptibility gene. Our limitation is that sample size in the present study is small. Bonferroni correction is effective at controlling experiment-wise α (αEW), but the correction is very conservative, and power (the proportion of the false null hypotheses that are correctly rejected) is greatly reduced\(^\text{23, 24}\). If we applied a Bonferroni correction for multiple testing to the results presented in Table 3, none of the results would show any significance. It may due to the small sample size. So larger population-based studies are needed to confirm these findings. Otherwise, it has recently been reported that it is more important to obtain confirmation in various populations than obtaining extremely low p-value in one single population\(^\text{25}\). Therefore, the association here should be confirmed through the analysis of other, independent noise-exposed populations.

The results in our study were similar to other studies. Peters et al. first reported that mutation of GRHL2 can cause DFNA28\(^\text{15}\). Moreover, in a recent population-based fine mapping study, GRHL2 was found to be highly associated with ARHL using 70 candidate genes with a total of 768 tagSNPs, in more than 2418 individuals from nine centers of seven European countries\(^\text{16}\). Recently, Han et al. revealed the conserved function of GRHL2 in otic development and established a model for further studying mechanisms of GRHL2-related hearing loss using the zebrafish\(^\text{18}\). As being both sensory impairment, although DFNA28 and ARHI do not match all the features observed in NIHL completely, but many properties totally correspond, such as the fact that the high frequencies are most affected, and the progressive and sensorineural nature\(^\text{16}\).

It is now believed that genes regulating the integrity and barrier function of the otic epithelial cells are critical to keep the homeostasis of the otic lumen, the perilymph and the endolymph, which is very important to normal conditions required for the development of the inner-ear structures, such as hair cells, otoliths and semicircular canals, as well as for the establishment of the mechanotransduction. Deficiency of the tight junction components could be harmful to the endolymph homeostasis, affects hair cell survival and otolith growth and causes deafness due to increased paracellular permeability in zebrafish\(^\text{26}\), mouse\(^\text{27}\) and human\(^\text{28}\). The GRHL2, as found to be a transcription factor, plays an essential role in epithelia morphogenesis and epidermal development in many types of organs and tissues in mice\(^\text{29–31}\) and zebrafish\(^\text{32}\). It can also regulate apical junctional proteins and the expression of desmosomal cadherin\(^\text{29}\) in mice and flies\(^\text{33}\). The extent of the impact of polymorphisms on hearing function and properties is under investigation\(^\text{12}\). Many studies showed that GRHL2 obtain functional diversity through the formation of homo- and heteromeric complexes and through the presence of tissue-specific isoforms. GRHL2 is known to homodimerize and to form heterodimers with the mammalian homologues GRHL1 and GRHL3\(^\text{34}\). Thus the information as to which homo- and/or heterodimers and GRHL2 isoforms are present in the inner ear is important\(^\text{16}\).

In conclusion, based on our current study, statistically significant association was found between the risk of NIHL and rs611419. Moreover, the combined genotypes
of these six polymorphisms were associated with risk of NIHL. In particular, the subjects carrying 3–8 variant alleles exhibited more resistant to NIHL compared with the workers carrying 0–2 variant alleles. These findings indicate that GRHL2 may be a NIHL susceptibility gene and the polymorphisms of GRHL2 may contribute to the etiology of NIHL. Larger population-based studies with different races are needed to confirm these findings.

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