

# Association between Genetic Variations in *GRHL2* and Noise-induced Hearing Loss in Chinese High Intensity Noise Exposed Workers: A Case-control Analysis

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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 *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 *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 an 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 *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

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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<sup>1-4</sup>). 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<sup>3</sup>).

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<sup>5</sup>). For example, many heterozygote and homozygote knockout mice studies identified that the gene coding for otocadherin 23 (*cdh23*)<sup>6</sup>), plasma membrane Ca<sup>2+</sup>-ATPase isoform 2 gene (*pmca2*)<sup>7</sup>), glutamate peroxidase 1 (*gpx1*)<sup>8</sup>), and heat shock factor (*hsf1*)<sup>9, 10</sup>) 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<sup>11-14</sup>).

The grainyhead like 2 (GRHL2), also known as BOM (brother of mammalian grainyhead) and TFCEP2L3 (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<sup>15</sup>). The underlying pathological reason of GRHL2's involvement in hearing loss such as ARHI is an impaired maintenance<sup>16</sup>). In *Drosophila*, grainyhead plays a critical role in the regulation of many important developmental processes, and the homozygous mutations in *grainyhead* are embryonic lethal<sup>17</sup>). Grainyhead-like family members (GRHL1-GRHL3) can regulate epithelial adhesion<sup>18</sup>).

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<sup>16, 18</sup>). 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)<sup>19</sup>). 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

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

Variables	Cases (n=340)		Controls (n=356)		<i>p</i> <sup>a</sup>
	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	

<sup>a</sup>Student's *t*-test for age, exposure level, exposure time and threshold distributions between cases and controls; two-sided  $\chi^2$  test for the other selected variables between cases and controls.

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.zybw.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.

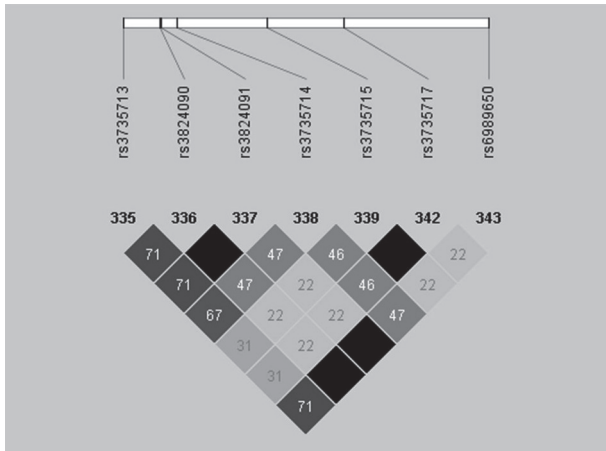
#### *Audiological assessment and definition of NIHL*

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*

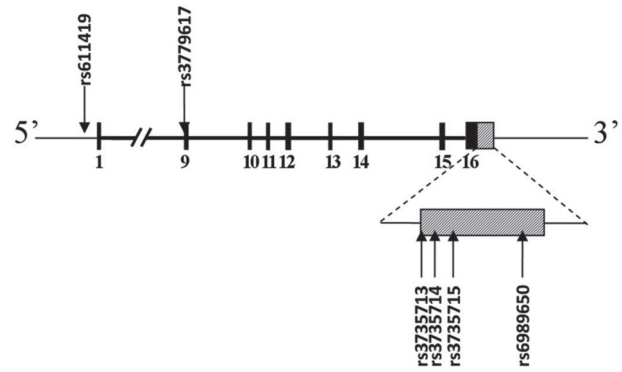
Recently, many genome-wide association studies (GWASs) have found that there might be some other functional polymorphisms located downstream or upstream



**Fig. 1. Pairwise Linkage Disequilibrium (LD) among seven SNPs in 3' untranslated region of the *GRHL2* gene.**

The LD plots were generated by Haploview 4.1. The value within each diamond represents the pairwise correlation ( $R^2$ ) between SNPs defined by the upper left and the upper right sides of the diamond. The diamonds without value mean the  $R^2=1.0$ , showed complete linkage disequilibrium.

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 ( $R^2$ ) for each pair of the seven SNPs in 3' UTR and found that rs3824090, rs3824091 and rs6989650 were in complete linkage disequilibrium (LD) ( $R^2=1.00$ ), while rs3735715 and rs3735717 were in LD too (Fig. 1). Thus the selection of rs6989650 and rs3735715 was enough<sup>20</sup>), 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 Tian-Gen 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



**Fig. 2. The location of the six SNPs in the *GRHL2* gene.**

The exons were indicated by black boxes and the untranslated regions were denoted by gray boxes.

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  $\chi^2$ -test. Demographic and genotype information for NIHL cases and controls were compared using the Student's *t*-test (for continuous variables) or  $\chi^2$ -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  $R^2$ , 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,

**Table 2.** Basic information of the six SNPs in *GRHL2* gene

SNPs (rs no.)	Base change	Location	MAF			<i>p</i> for HWE <sup>b</sup>	Genotyped (%)
			HapMap <sup>a</sup>	Case	Control		
rs611419	A>T	5' near gene	0.356	0.425	0.482	0.613	99.9
rs3779617	G>A	Exon 9	0.116	0.090	0.103	0.316	99.9
rs3735713	G>A	3'UTR	0.302	0.297	0.309	0.806	100.0
rs3735714	C>T	3'UTR	0.389	0.379	0.392	0.883	99.9
rs3735715	G>A	3'UTR	0.422	0.404	0.438	0.153	100.0
rs6989650	C>T	3'UTR	0.233	0.208	0.222	0.652	99.9

<sup>a</sup>MAF from the HapMap database (<http://www.hapmap.org>). <sup>b</sup>Goodness-of-fit  $\chi^2$  test for Hardy-Weinberg equilibrium in the controls.

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 statuses ( $p=0.927$  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).

#### *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).

#### *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 risk. The subjects who carrying 3–8 variant alleles were more resistant to NIHL compared with those carrying 0–2 variant alleles. And this protective effect was more evident for subgroup of long time exposure and drinkers. These results suggested that the selected six polymorphisms of *GRHL2* may affect



**Table 3. Genotype and allele frequencies of the *GRHL2* polymorphisms among the NIHL cases and controls and the associations with risk of NIHL**

Genotypes	Cases (n=340)		Controls (n=356)		<i>P</i> <sup>a</sup>	Adjusted OR (95%CI) <sup>b</sup>
	n	%	n	%		
rs611419	339		356			
AA	118	34.8	98	27.5	0.097	1.00 (reference)
AT	154	45.4	173	48.6		0.74 (0.52–1.04)
TT	67	19.8	85	23.9		0.66 (0.44–1.01)
<i>P</i> <sub>trend</sub>					0.038	
AT/TT	221	65.2	258	72.5	0.038	0.71 (0.52–0.98)
T allele	0.425		0.482		0.033	
rs3779617	339		356			
GG	279	82.3	285	80.1	0.683	1.00 (reference)
AG	59	17.4	69	19.4		0.89 (0.60–1.30)
AA	1	0.3	2	0.5		0.77 (0.07–8.96)
<i>P</i> <sub>trend</sub>					0.414	
AG/AA	60	17.7	71	19.9	0.450	0.88 (0.60–1.29)
A allele	0.09		0.103		0.428	
rs3735713	340		356			
GG	172	50.6	169	47.4	0.588	1.00 (reference)
AG	134	39.4	154	43.3		0.84 (0.61–1.15)
AA	34	10	33	9.3		0.99 (0.58–1.67)
<i>P</i> <sub>trend</sub>					0.632	
AG/AA	168	49.4	187	52.6	0.411	0.87 (0.64–1.17)
A allele	0.297		0.309		0.628	
rs3735714	339		356			
CC	134	39.5	131	36.8	0.720	1.00 (reference)
CT	153	45.2	171	48		0.87 (0.63–1.21)
TT	52	15.3	54	15.2		0.92 (0.58–1.45)
<i>P</i> <sub>trend</sub>					0.627	
CT/TT	205	60.5	225	63.2	0.459	0.87 (0.64–1.19)
T allele	0.379		0.392		0.624	
rs3735715	340		356			
GG	122	35.8	119	33.4	0.345	1.00 (reference)
AG	161	47.4	162	45.5		0.99 (0.71–1.38)
AA	57	16.8	75	21.1		0.73 (0.47–1.14)
<i>P</i> <sub>trend</sub>					0.213	
AG/AA	218	64.2	237	66.6	0.496	0.91 (0.67–1.25)
A allele	0.404		0.438		0.202	
rs6989650	339		356			
CC	212	62.5	217	61	0.737	1.00 (reference)
CT	113	33.3	120	33.7		0.93 (0.68–1.29)
TT	14	4.2	19	5.3		0.73 (0.35–1.50)
<i>P</i> <sub>trend</sub>					0.529	
CT/TT	127	37.5	139	39	0.668	0.92 (0.67–1.25)
T allele	0.208		0.222		0.527	

<sup>a</sup>Two-sided  $\chi^2$  test for the distributions of genotype frequencies. <sup>b</sup>Adjusted for age, sex, exposure level and exposure time in logistic regression model.

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

**Table 4. Frequency distributions of the combined genotypes of the *GRHL2* polymorphisms between the cases and controls and the associations with risk of NIHL**

Numbers of variants <sup>a</sup>	Cases (n=337)		Controls (n=356)		<i>p</i> <sup>b</sup>	Adjusted OR (95%CI) <sup>c</sup>
	n	%	n	%		
0	7	2.1	1	0.3	0.029	
1	32	9.5	20	5.6		
2	47	14	46	12.9		
3	68	20.2	90	25.3		
4	85	25.2	83	23.3		
5	53	15.7	58	16.3		
6	33	9.8	30	8.4		
7	11	3.3	23	6.5		
8	1	0.3	5	1.4		
Combined genotypes						
0–2	86	25.5	67	18.8	0.034	1.00 (reference)
3–8	251	74.5	289	81.2		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  $\chi^2$  test for the distributions of genotype frequencies. <sup>c</sup>Adjusted for age, sex, exposure level and exposure time in logistic regression model.

**Table 5. Stratification analyses between the combined genotypes of the *GRHL2* polymorphisms and risk of NIHL**

Variables	Cases/controls	Combined genotypes (cases/controls)		<i>p</i> <sup>a</sup>	Adjusted OR (95%CI) <sup>b</sup>
		0–2	3–8		
Age					
<40	171/165	44/33	127/132	0.212	0.71 (0.43–1.19)
≥40	166/191	42/34	124/157	0.084	0.64 (0.39–1.07)
Gender					
Male	303/317	73/58	230/259	0.077	0.71 (0.48–1.04)
Female	34/39	41/30	21/30	0.159	0.55 (0.19–1.62)
Exposure time (years)					
≤20	233/248	51/52	182/196	0.806	0.93 (0.60–1.44)
>20	104/108	35/15	69/93	0.001	0.31 (0.16–0.62)
Smoking status					
Non-smokers	143/152	35/25	108/127	0.087	0.60 (0.34–1.08)
Smokers	194/204	51/42	143/162	0.179	0.72 (0.45–1.15)
Drinking status					
Non-drinkers	184/198	47/44	137/154	0.446	0.84 (0.52–1.35)
Drinkers	153/158	39/23	114/135	0.016	0.51 (0.29–0.90)

<sup>a</sup>Two-sided  $\chi^2$  test for the distributions of genotype frequencies. <sup>b</sup>Adjusted for age, sex, exposure level and exposure time in logistic regression model.

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 individu-

als 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

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)<sup>21)</sup>. 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<sup>12)</sup>. 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<sup>22)</sup>. 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  $\alpha$  ( $\alpha_{EW}$ ), but the correction is very conservative, and power (the proportion of the false null hypotheses that are correctly rejected) is greatly reduced<sup>23, 24)</sup>. 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<sup>25)</sup>. 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.* firstly reported that mutation of *GRHL2* can cause DFNA28<sup>15)</sup>. Moreover, in a recent population-based fine mapping study, *GRHL2* was found to be highly associated with ARHI, using 70 candidate genes with a total of 768 tagSNPs, in more than 2418 individuals from nine centers of seven European countries<sup>16)</sup>. 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<sup>18)</sup>. 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<sup>16)</sup>.

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 zabrafish<sup>26)</sup>, mouse<sup>27)</sup> and human<sup>28)</sup>. 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<sup>29–31)</sup> and zabrafish<sup>32)</sup>. It can also regulate apical junctional proteins and the expression of desmosomal cadherin<sup>29)</sup> in mice and flies<sup>33)</sup>. The extent of the impact of polymorphisms on hearing function and properties is under investigation<sup>12)</sup>. 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*<sup>34)</sup>. Thus the information as to which homo- and/or heterodimers and *GRHL2* isoforms are present in the inner ear is important<sup>16)</sup>.

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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## References

- Campo P, Lataye R (2000) Noise and solvent, alcohol and solvent: two dangerous interactions on auditory function. *Noise Health* **3**, 49–57.
- Fechter LD, Chen GD, Rao D (2002) Chemical Asphyxiants and Noise. *Noise Health* **4**, 49–61.
- Sliwinska-Kowalska M (2008) Organic solvent exposure and hearing loss. *Occup Environ Med* **65**, 222–3.
- Ward WD (1995) Endogenous factors related to susceptibility to damage from noise. *Occup Med* **10**, 561–75.
- Borg E, Canlon B, Engstrom B (1995) Noise-induced hearing loss. Literature review and experiments in rabbits. Morphological and electrophysiological features, exposure parameters and temporal factors, variability and interactions. *Scand Audiol Suppl* **40**, 1–147.
- Holme RH, Steel KP (2004) Progressive hearing loss and increased susceptibility to noise-induced hearing loss in mice carrying a *Cdh23* but not a *Myo7a* mutation. *J Assoc Res Otolaryngol* **5**, 66–79.
- Kozel PJ, Davis RR, Krieg EF, Shull GE, Erway LC (2002) Deficiency in plasma membrane calcium ATPase isoform 2 increases susceptibility to noise-induced hearing loss in mice. *Hear Res* **164**, 231–9.
- Ohlemiller KK, McFadden SL, Ding DL, Lear PM, Ho YS (2000) Targeted mutation of the gene for cellular glutathione peroxidase (*Gpx1*) increases noise-induced hearing loss in mice. *J Assoc Res Otolaryngol* **1**, 243–54.
- Fairfield DA, Lomax MI, Dootz GA, Chen S, Galecki AT, Benjamin IJ, Dolan DF, Altschuler RA (2005) Heat shock factor 1-deficient mice exhibit decreased recovery of hearing following noise overstimulation. *J Neurosci Res* **81**, 589–96.
- Sugahara K, Inouye S, Izu H, Katoh Y, Katsuki K, Takemoto T, Shimogori H, Yamashita H, Nakai A (2003) Heat shock transcription factor HSF1 is required for survival of sensory hair cells against acoustic overexposure. *Hear Res* **182**, 88–96.
- Konings A, Van Laer L, Pawelczyk M, Carlsson PI, Bondeson ML, Rajkowska E, Dudarewicz A, Vandeveld A, Fransen E, Huyghe J, Borg E, Sliwinska-Kowalska M, Van Camp G (2007) Association between variations in *CAT* and noise-induced hearing loss in two independent noise-exposed populations. *Hum Mol Genet* **16**, 1872–83.
- Konings A, Van Laer L, Wiktorek-Smagur A, Rajkowska E, Pawelczyk M, Carlsson PI, Bondeson ML, Dudarewicz A, Vandeveld A, Fransen E, Huyghe J, Borg E, Sliwinska-Kowalska M, Van Camp G (2009) Candidate gene association study for noise-induced hearing loss in two independent noise-exposed populations. *Ann Hum Genet* **73**, 215–24.
- Liu YM, Li XD, Guo X, Liu B, Lin AH, Rao SQ (2010) Association between polymorphisms in *SOD1* and noise-induced hearing loss in Chinese workers. *Acta Otolaryngol* **130**, 477–86.
- Yang M, Tan H, Yang Q, Wang F, Yao H, Wei Q, Tanguay RM, Wu T (2006) Association of *hsp70* polymorphisms with risk of noise-induced hearing loss in Chinese automobile workers. *Cell Stress Chaperones* **11**, 233–9.
- Peters LM, Anderson DW, Griffith AJ, Grundfast KM, San Agustin TB, Madeo AC, Friedman TB, Morell RJ (2002) Mutation of a transcription factor, *TFCP2L3*, causes progressive autosomal dominant hearing loss, *DFNA28*. *Hum Mol Genet* **11**, 2877–85.
- Van Laer L, Van Eyken E, Fransen E, Huyghe JR, Topsakal V, Hendrickx JJ, Hannula S, Maki-Torkko E, Jensen M, Demeester K, Baur M, Bonaconsa A, Mazzoli M, Espeso A, Verbruggen K, Huyghe J, Huygen P, Kunst S, Manninen M, Konings A, Diaz-Lacava AN, Steffens M, Wienker TF, Pyykko I, Cremers CW, Kremer H, Dhooge I, Stephens D, Orzan E, Pfister M, Bille M, Parving A, Sorri M, Van de Heyning PH, Van Camp G (2008) The grainyhead like 2 gene (*GRHL2*), alias *TFCP2L3*, is associated with age-related hearing impairment. *Hum Mol Genet* **17**, 159–69.
- Wilanowski T, Tuckfield A, Cerruti L, O'Connell S, Saint R, Parekh V, Tao J, Cunningham JM, Jane SM (2002) A highly conserved novel family of mammalian developmental transcription factors related to *Drosophila* grainyhead. *Mech Dev* **114**, 37–50.
- Han Y, Mu Y, Li X, Xu P, Tong J, Liu Z, Ma T, Zeng G, Yang S, Du J, Meng A (2011) *Grlh2* deficiency impairs otic development and hearing ability in a zebrafish model of the progressive dominant hearing loss *DFNA28*. *Hum Mol Genet* **20**, 3213–26.

- 19) Acoustics—Determination of occupational noise exposure and estimation of noise-induced hearing impairment. [http://www.iso.org/iso/home/store/catalogue\\_tc/catalogue\\_detail.htm?csnumber=6759](http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=6759). Accessed March 13, 2013.
- 20) Wei Y, Wang L, Lan P, Zhao H, Pan Z, Huang J, Lu J, Wang J (2009) The association between -1304T>G polymorphism in the promoter of MKK4 gene and the risk of sporadic colorectal cancer in southern Chinese population. *Int J Cancer* **125**, 1876–83.
- 21) Lutman ME (2000) What is the risk of noise-induced hearing loss at 80, 85, 90 dB(A) and above? *Occup Med (Lond)* **50**, 274–5.
- 22) Marchini J, Donnelly P, Cardon LR (2005) Genome-wide strategies for detecting multiple loci that influence complex diseases. *Nat Genet* **37**, 413–7.
- 23) Ryman N, Jorde PE (2001) Statistical power when testing for genetic differentiation. *Mol Ecol* **10**, 2361–73.
- 24) García LV (2004) Escaping the Bonferroni iron claw in ecological studies. *Oikos* **105**, 657–63.
- 25) Neale BM, Sham PC (2004) The future of association studies: gene-based analysis and replication. *Am J Hum Genet* **75**, 353–62.
- 26) Hardison AL, Lichten L, Banerjee-Basu S, Becker TS, Burgess SM (2005) The zebrafish gene *claudinj* is essential for normal ear function and important for the formation of the otoliths. *Mech Dev* **122**, 949–58.
- 27) Nakano Y, Kim SH, Kim HM, Sanneman JD, Zhang Y, Smith RJ, Marcus DC, Wangemann P, Nessler RA, Banfi B (2009) A claudin-9-based ion permeability barrier is essential for hearing. *PLoS Genet* **5**, e1000610.
- 28) Bashir R, Fatima A, Naz S (2010) Mutations in CLDN14 are associated with different hearing thresholds. *J Hum Genet* **55**, 767–70.
- 29) Wilanowski T, Caddy J, Ting SB, Hislop NR, Cerruti L, Auden A, Zhao LL, Asquith S, Ellis S, Sinclair R, Cunningham JM, Jane SM (2008) Perturbed desmosomal cadherin expression in grainy head-like 1-null mice. *EMBO J* **27**, 886–97.
- 30) Yu Z, Mannik J, Soto A, Lin KK, Andersen B (2009) The epidermal differentiation-associated Grainyhead gene *Get1/Grhl3* also regulates urothelial differentiation. *EMBO J* **28**, 1890–903.
- 31) Yu Z, Bhandari A, Mannik J, Pham T, Xu X, Andersen B (2008) Grainyhead-like factor *Get1/Grhl3* regulates formation of the epidermal leading edge during eyelid closure. *Dev Biol* **319**, 56–67.
- 32) Janicke M, Renisch B, Hammerschmidt M (2010) Zebrafish grainyhead-like1 is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a *Foxi3*-dependent manner. *Int J Dev Biol* **54**, 837–50.
- 33) Werth M, Walentin K, Aue A, Schonheit J, Wuebken A, Pode-Shakked N, Vilianovitch L, Erdmann B, Dekel B, Bader M, Barasch J, Rosenbauer F, Luft FC, Schmidt-Ott KM (2010) The transcription factor grainyhead-like 2 regulates the molecular composition of the epithelial apical junctional complex. *Development* **137**, 3835–45.
- 34) Ting SB, Wilanowski T, Cerruti L, Zhao LL, Cunningham JM, Jane SM (2003) The identification and characterization of human Sister-of-Mammalian Grainyhead (SOM) expands the grainyhead-like family of developmental transcription factors. *Biochem J* **370**, 953–62.