

***GRHL2* genetic polymorphisms may confer a protective effect against sudden sensorineural hearing loss**

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Abstract. Genetic polymorphisms in grainyhead-like 2 (*GRHL2*) variants were examined for their suspected association with sudden sensorineural hearing loss (SSHL). Between January 2009 and April 2014, 190 patients with SSHL, who were diagnosed at the Departments of Otorhinolaryngology Head and Neck Surgery at Kaihua People's Hospital and Hangzhou First People's Hospital, were selected for the present study and defined as the SSHL group. A group of 210 healthy individuals were defined as the control group. Polymerase chain reaction (PCR)-restriction fragment length polymorphism was used to detect *GRHL2* genotypes, using genomic DNA isolated from peripheral blood as PCR templates. *GRHL2* rs611419 genetic polymorphisms conferred a protective effect against SSHL (AT+TT vs. AA: OR=0.63, 95% CI=0.41-0.98, P=0.038). In addition, rs10955255 polymorphisms were associated with a reduced risk of SSHL (AA vs. GG: OR=0.54, 95% CI=0.31-0.95, P=0.032; GA+AA vs. GG: OR=0.58, 95% CI=0.38-0.89, P=0.012). Combined genotypes of rs611419, rs10955255 and rs6989650 in the *GRHL2* gene are also associated with a reduced risk of SSHL (P=0.035). In subjects who consumed alcohol, co-occurrence of 3-8 variant alleles conferred increased resistance to SSHL, compared with the occurrence of 0-2 variant alleles (OR=0.40, 95% CI=0.21-0.76, P=0.004). *GRHL2* genetic polymorphisms, rs611419 and rs10955255, have a protective role against SSHL and reduce the risk of SSHL. However, rs6989650 is not associated with SSHL.

Introduction

Sudden sensorineural hearing loss (SSHL) is defined as a sensorineural loss of hearing function, generally in one ear, occurring over a short period of time due to uncertain causes (1). SSHL is characterized by a loss of >30 dB in at least three audiometric frequencies over a period of 12-72 h or more (2). SSHL predominantly occurs in age groups ranging between 50 and 60 years and the morbidity, due to varied underlying causes, reaches 5-20 per 100,000 individuals each year with no difference in gender or region (3). The risk factors of SSHL include hypertension, hypotension, diabetes mellitus, stroke and acquired and inherited cardiopathy (3,4). Unhealthy lifestyle habits, including smoking, alcohol consumption, a sedentary lifestyle and sleep deprivation can also lead to SSHL (5,6), however, the exact etiology of SSHL remains to be elucidated. Previous studies have identified a few underlying events, including vascular compromise, cochlear membrane rupture and viral infection, in SSHL (7-9). In previous years, genetic predisposition to SSHL susceptibility has been actively investigated. In this context, nitric oxide synthase 3, caveolin 1 and grainyhead-like 2 (*GRHL2*) are suggested to be involved in the etiology of SSHL (10-12).

GRHL2, also called brother of mammalian grainyhead or transcription factor cellular promoter 2-like 3, is a transcription factor that belongs to the grainyhead-like family (13). *GRHL2* was initially identified in *Drosophila* and is important in the organization of septate junctions, and thus is critical for maintaining apical barrier functions in the epithelium (14). In humans, *GRHL2* is also predominantly expressed in the epithelial tissues and is important in embryonic development, terminal differentiation of epithelial cells, establishment and maintenance of human mucociliary airway epithelium and neural tube closure (15,16). Multiple diseases are associated with *GRHL2*, including gastric diseases, breast cancer and sensorineural hearing loss (SHL) (15-17). The *GRHL2* gene in humans is located on chromosome 8q22.3, and includes 16 exons and 15 introns (15,18). Genetic polymorphisms in *GRHL2* are associated with the development of SHL, including noise-induced hearing loss (NIHL) and age-related hearing impairment (ARHI) (15,19,20). However, the connection between the *GRHL2* gene and susceptibility to SSHL, which is a very important category of SHL, has not been thoroughly investigated.

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In the present study, SSHL patients and healthy individuals were compared for genetic variations in *GRHL2*. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to detect *GRHL2* genotypes. The polymorphisms in *GRHL2* and their association with susceptibility to SSHL in a Chinese population was thus investigated in order to determine the etiology of SSHL and to provide valuable clinical diagnostic tools for SSHL.

Subjects and methods

Study subjects. Between January 2009 and April 2014, 190 patients with SSHL, referred to the Departments of Otorhinolaryngology Head and Neck Surgery at Kaihua People's Hospital (Quzhou, China) and Hangzhou First People's Hospital (Hangzhou, China), were selected, and included 108 males and 82 females with an average age of 38.5 ± 4.8 years. A total of 210 age- and gender-matched healthy subjects were selected, including 115 males and 95 females with an average age of 38.9 ± 5.3 years. Pure-tone audiometry was performed and tobacco smoking and alcohol consumption status were recorded in all participants. The selection criteria were based on the Sudden Sensorineural Hearing Loss Diagnosis and Treatment Guidelines published by the Chinese Medical Association Archives of Otolaryngology Head and Neck Surgery Branch (21). The diagnostic standards were as follows: SSHL occurring in a few minutes, hours or within 3 days; non fluctuating sensorineural hearing loss (categorized into mild, moderate severe or even complete deafness), sensorineural hearing loss of 20 dB or more over three contiguous audiometric frequencies; unknown cause due to systemic or local factors; accompanying with tinnitus, ear blockage sensation, and non-recurrent dizziness, nausea and vomiting; no other cranial nerve injury with the exception of eighth cranial nerve injury (http://d.wanfangdata.com.cn/Periodical_zhebyhk200608003.aspx). The study protocol was approved by the Institutional Review Board of the Kaihua People's Hospital (Quzhou, China). Written informed consent was signed by each subject.

DNA extraction from blood clots. A commercially available blood DNA purification kit (Beijing CWBio Co., Ltd., Beijing, China) was used to extract genomic DNA from blood clots. The eluted DNA solution was collected and stored at -20°C .

DNA content and purity detection. A UV 260 spectrophotometer (Shimadzu Corp., Kyoto, Japan) was used to measure the absorbance at wavelengths of 260 and 280 nm. The Lambert-Beer law was used to calculate sample concentration based on the $c = A_{260}/(\epsilon \times b)$ equation, where ϵ is the molar absorption coefficient, b is the optical path length and c is the molar concentration. The A_{260}/A_{280} ratio was used to determine the sample purity. DNA concentration was adjusted at $>15 \text{ ng}/\mu\text{l}$ and the purity of DNA was between 1.6 and 1.9.

Selection of *GRHL2* SNPs. SNPs of the human *GRHL2* gene on chromosome 8 (8q22.3) were retrieved and the data packet was downloaded from NCBI-dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) and HapMap (http://SNP.cshl.org/cgi-Perl/gbrowse/haPmaP27_B36/databases). Haploview 4.2 (<http://www.broad.mit.edu/mpg/haploview/>)

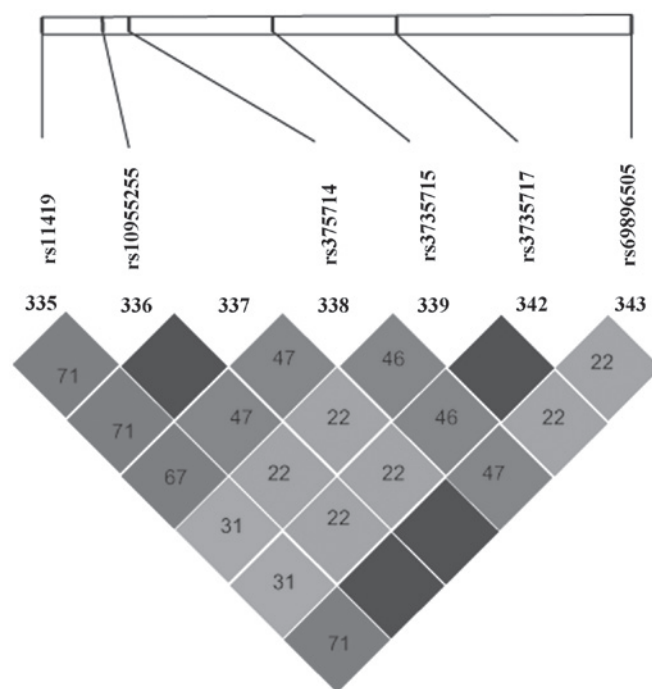


Figure 1. Pairwise linkage disequilibrium among three single nucleotide polymorphisms in the 3'untranslated region of the grainyhead-like 2 gene.

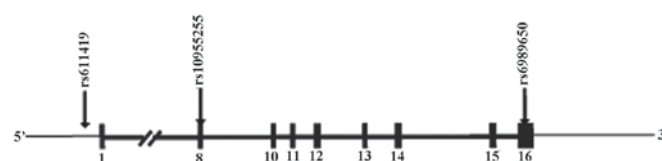


Figure 2. Location of three single nucleotide polymorphisms rs611419, rs10955255 and rs6898650 in the grainyhead-like 2 gene.

statistical software was used to select the tag SNPs of *GRHL2* and the parameters were set for Han Chinese in Beijing with a minor allele frequency (MAF) $>10\%$, $r^2 > 0.8$, $D' = 1$. The base sequences of the selected site were suitable for primers designed for PCR amplification. Three tag SNPs termed rs611419, rs10955255 and rs6898650, were selected to stand for 100% SNP sites of the *GRHL2* gene (Fig. 1). The loci of the three SNPs is present in Fig. 2.

Primer design. The PCR amplification primers for the three SNPs were designed using Assay Designer 3.1 software (Sequenom, San Diego, CA, USA) and their feasibility was based on the following criteria: i) Primers and the templates should be complementary; ii) stable dimers or hairpins between primers should be avoided; iii) DNA mismatch with the template at non-target sites should be avoided. Upstream and downstream primers for PCR reaction should strictly comply with the design principles of primers. Three primers are shown in Table I.

Genotyping. PCR-RFLP was used to determine the SNP genotype. PCR reaction conditions were as follows: 5 min initial denaturation at 94°C , 36 cycles of 30 sec denaturation at 94°C , 40 sec annealing at 60°C and 45 sec extension at 72°C , followed by 7 min extension at 72°C . The amplification products

Table I. Primers for PCR amplification of *GRHL2* gene polymorphisms at rs611419, rs10955255 and rs6898650.

SNP	Primers for PCR amplification (5'-3')	Length (nt)	Molecular weight (g/M)
rs611419	F: 5'-GGAAATACTGGCACTCTCG-3'	19	5,809
	R: 5'-ACCTTCTCGTTCATCATCC-3'	19	5,650
rs10955255	F: 5'-CCGTGAATTGCTTGAGCACA-3'	20	6,113
	R: 5'-GGTTTGCAAAGTGAACATCAG-3'	21	6,489
rs6898650	F: 5'-GGATTTCACCTGGTTTATAGG-3'	19	5,884
	R: 5'-AGCGTAGACTTCAAGTGAGC-3'	20	6,160

PCR, polymerase chain reaction; *GRHL2*, grainyhead-like 2; SNP, single nucleotide polymorphism; F, forward; R, reverse.

were isolated and identified by agarose gel electrophoresis at a voltage of 120 V for 30 min. PCR products (15 μ l) were digested with shrimp alkaline phosphatase and the restriction enzyme *ExoI* at 60°C for 37 min and at 75°C for 15 min, respectively. The resulting restriction fragments were electrophoresed in 2% agarose gels and visualized under UV light. An ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used to compare the resulting restriction fragments and PCR products to identify the genotype.

Statistical analysis. SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA) was applied to analyze the data. Hardy-Weinberg equilibrium was used to confirm the sample representation in the population. Data are expressed as the mean \pm standard deviation and χ^2 test was used to compare group differences. Logistic regression analysis was used to calculate the odds ratios and 95% confidence interval of each genotype was used to represent the relative risk. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data in the SSHL group and the control group. Table II shows the profiles of the SSHL group and the control group. The auditory threshold in the SSHL group (36.6 \pm 10.5 dB) was significantly higher than the control group (13.2 \pm 3.0 dB) ($P < 0.01$). No significant differences in age, gender, smoking status and drinking status were found between the SSHL group and the control group.

Basic information of the three SNPs in the *GRHL2* gene. Table III describes the basic information of rs611419, rs10955255 and rs6898650. rs611419 is located in the 5' region with the A allele and T allele. rs10955255 is located in intron 8 with the G allele and A allele. rs6898650 is located in the 3'UTR with the C allele and T allele. χ^2 goodness-of-fit test was used to detect the genotype frequency distribution of rs611419, rs10955255 and rs6898650 in the SSHL group and the control group, and the results demonstrated that the three sites in the two groups conformed to the Hardy-Weinberg equilibrium (all $P > 0.05$).

Genotype and allele frequency distributions in the *GRHL2* gene. Table IV shows the results from logistic regression regarding the risk of SSHL. rs611419 of the *GRHL2* gene may

Table II. Characteristics of SSHL patients and controls.

Variable	SSHL group	Control group	P-value ^a
Age (years)	38.5 \pm 4.8	38.9 \pm 5.3	0.431
<40 (n, %)	96 (50.5)	98 (46.7)	0.441
\geq 40 (n, %)	94 (49.5)	112 (53.3)	
Gender			
Male (n, %)	108 (56.8)	115 (54.8)	0.676
Female (n, %)	82 (43.2)	95 (45.2)	
Threshold (dB)	36.6 \pm 10.5	13.2 \pm 3.0	<0.0001
Smoking status			
Non-smoker (n, %)	80 (42.1)	90 (42.9)	0.879
Smoker (n, %)	110 (57.9)	120 (57.1)	
Drinking status			
Non-drinker (n, %)	105 (55.3)	116 (55.2)	0.996
Drinker (n, %)	85 (44.7)	94 (44.8)	

Student's t-test for age and threshold distributions between the SSHL group and the control group; two-sided χ^2 test for gender and drinking and smoking status between the SSHL group and the control group. Age is presented as mean \pm standard deviation. ^aP-values indicates the statistically significant differences between the SSHL group and the control group. SSHL, sudden sensorineural hearing loss.

be a protective factor for SSHL (AT+TT vs. AA: OR=0.63, 95% CI=0.41-0.98, $P=0.038$). Similarly, rs10955255 site polymorphisms may reduce the risk of SSHL (AA vs. GG: OR=0.54, 95% CI=0.31-0.95, $P=0.32$; GA+AA vs. GG: OR=0.58, 95% CI=0.38-0.89, $P=0.012$). However, genotype and allele frequencies of rs6898650 demonstrated no statistical differences in the SSHL group and the control group ($P > 0.05$).

Combined analysis between the three SNPs and SSHL risk. In order to examine the interaction between these polymorphisms, the three polymorphisms were combined for the analysis. There was a marked association between the combined genotypes and SSHL risk ($P=0.035$). Subjects carrying 3-8 variant alleles demonstrated a lower SSHL risk compared with subjects carrying 0-2 variant alleles (OR=0.59, 95% CI=0.36-0.96, $P=0.034$; Table V).

Table III. Basic information and HWE results of the three SNPs in the *GRHL2* gene.

SNP (rs no.)	Base change	Location	MAF			HWE(χ^2/P)	
			HapMap ^a	SSHL group	Control group	SSHL group	Control group
rs611419	A>T	5' gene site	0.356	0.425	0.482	0.048/0.826	0.075/0.785
rs10955255	G>A	Intron 8	0.116	0.090	0.103	1.305/0.253	0.081/0.776
rs6989650	C>T	3'UTR	0.233	0.208	0.222	0.224/0.636	1.343/0.247

^aMAF from the HapMap database (<http://www.hapmap.org>). SNP, single nucleotide polymorphism; MAF, minor allele frequency; 3'UTR, 3' untranslated region; SSLH, sudden sensorineural hearing loss; HWE, Hardy-Weinberg equilibrium; *GRHL2*, grainyhead-like 2.

Table IV. Association of genotypes and allele frequencies of the *GRHL2* genetic polymorphisms and risk of SSLH.

Genotype	SSHL group (n=190)		Control group (n=210)		P-value ^a	OR (95% CI) ^b
	n	%	n	%		
rs611419						
AA	65	34.1	52	24.8	0.046	1.00 (Ref.)
AT	91	48.1	103	49.0	0.140	0.71 (0.45-1.12)
TT	34	17.8	55	26.2	0.014	0.49 (0.28-0.87)
AT+TT	125	65.9	158	75.2	0.038	0.63 (0.41-0.98)
rs10955255						
GG	71	37.5	54	25.7	0.040	1.00 (Ref.)
AG	84	44.1	107	51.0	0.026	0.60 (0.38-0.94)
AA	35	18.4	49	23.3	0.032	0.54 (0.31-0.95)
AG+AA	119	62.5	156	74.3	0.012	0.58 (0.38-0.89)
rs6989650						
CC	123	64.7	134	63.8	0.462	1.00 (Ref.)
CT	61	32.2	64	30.6	0.863	1.04 (0.68-1.59)
TT	6	3.1	12	5.6	0.233	0.54 (0.20-1.50)
CT+TT	67	35.3	76	36.2	0.847	0.96 (0.64-1.45)

^aTwo-sided χ^2 test for the distributions of genotype frequencies. ^bAdjusted for age and gender in the logistic regression model. SSLH, sudden sensorineural hearing loss; OR, odds ratio; CI, confidence interval; *GRHL2*, grainyhead-like 2; Ref., wild homozygous genotype of each site was used as a reference.

Stratification analysis of the combined genotypes of the GRHL2 polymorphisms and SSLH risk. Stratification analysis based on age, gender, smoking status and drinking status was conducted to analyze the effect of the combined genotypes of the three SNPs on SSLH risk. In individuals who consumed alcohol, subjects carrying 3-8 variant alleles were more resistant to SSLH compared with subjects carrying 0-2 variant alleles (OR=0.40, 95% CI=0.21-0.76, P=0.004). However, no significant differences were found between the frequency of combined genotypes and age, gender and smoking status (all P>0.05; Table VI).

Discussion

The etiology of SSLH remains to be elucidated. Vascular occlusion is a potential cause of impaired cochlear perfusion, and risk factors, including factor V Leiden and prothrombin

G20210A that lead to vascular perfusion defects, are also suspected to be important in SSLH (22-24). By contrast, inner ear damage was also associated with SSLH and Yamamoto *et al* reported that insulin-like growth factor-1, a growth factor involved in the development of the human inner ear, enhanced the regeneration of hair cells damaged in SSLH (25). Genetic factors associated with SSLH have generated significant interest. The present study investigated the association between *GRHL2* genetic polymorphisms and susceptibility to SSLH. The exact protective function of *GRHL2* in reducing susceptibility to SSLH remains to be elucidated. As a transcription factor, *GRHL2* is important in embryonic development and otic epithelial tissue differentiation by promoting apical junction maturation (14,26,27). *GRHL2* could also be involved in apical barrier formation and activate adult antimicrobial defense, which may be associated with SSLH observed in viral infections (28,29). *GRHL2* could

Table V. Association of frequency distribution of the combined genotypes of *GRHL2* polymorphisms and the risk of SSHL.

Number of variants ^a	SSHL group (n=190)		Control group (n=210)		P-value ^b	OR (95% CI) ^c
	n	%	n	%		
0	4	2.1	1	0.5	0.035	
1	18	9.5	8	3.8		
2	25	13.2	25	11.9		
3	34	17.9	60	28.6		
4	39	20.5	45	21.4		
5	43	22.6	37	17.6		
6	27	14.2	34	16.2		
Combined genotype						
0-2	47	24.7	34	16.2	0.034	1.00 (Ref.)
3-8	143	75.3	176	83.8		0.59 (0.36-0.96)

^a0-8 represents the number of variants within the combined genotypes; the variant alleles used for the calculation were rs611419T, rs10955255A and rs6989650T; 0-2=0-2 variant alleles. ^bTwo-sided χ^2 test for the distributions of genotype frequencies. ^cAdjusted for age and gender in the logistic regression model. SSHL, sudden sensorineural hearing loss; OR, odds ratio; CI, confidence interval; *GRHL2*, grainyhead-like 2.

Table VI. Stratification analyses between the combined genotypes of the *GRHL2* polymorphisms and risk of SSHL.

Variable	SSHL/control group	Combined genotypes		P-value ^a	OR (95% CI) ^b
		0-2	3-8		
Age (years)					
<40	96/98	21/13	75/85	0.115	1.83 (0.86-3.91)
≥40	94/112	26/21	68/91	0.129	1.66 (0.86-3.19)
Gender					
Male	108/115	36/28	133/159	0.121	1.54 (0.89-2.65)
Female	82/95	11/6	10/17	0.074	3.12 (0.88-11.04)
Smoking status					
Non-smoker	80/90	20/14	60/76	0.124	1.81 (0.84-3.88)
Smoker	110/120	27/20	83/100	0.139	1.63 (0.85-3.11)
Drinking status					
Non-drinker	105/116	21/13	75/38	0.621	0.82 (0.37-1.81)
Drinker	85/94	26/21	68/138	0.004	0.40 (0.21-0.76)

^aTwo-sided χ^2 test for the distribution of genotype frequencies. ^bAdjusted for age and gender in logistic regression model. SSHL, sudden sensorineural hearing loss; OR, odds ratio; CI, confidence interval; *GRHL2*, grainyhead-like 2.

also regulate the expression of *Rho GEF 19*, which is involved in wound healing, and thus the protective role of *GRHL2* may involve tissue repair processes (30).

In the present study, SSHL patients with the rs611419 AT/TT genotype demonstrated a lower susceptibility to SSHL than patients with the AA genotype, suggesting that the T allele in rs611419 polymorphism may be a protective factor to SSHL in the Chinese population. In addition, the significant difference in SSHL risk between genotype AA and GG in rs10955255 demonstrated that the A allele in rs10955255 reduced the risk of SSHL. Nevertheless, no association between the rs6989650 polymorphism and SSHL was identified, suggesting that rs6989650 may not be a risk factor

for SSHL. Van Laer *et al* analyzed 703 SNPs and found that rs10955255 and rs2127034 in *GRHL2* are ranked the top two in association with ARHI (13). In addition, Li *et al* also observed that rs611419 was significantly associated with NIHL (15), in agreement with the results of the present study.

In the present study, a marked association was found between the combined genotypes and SSHL risk. The results demonstrated that the combined genotypes correlated with lower SSHL incidence and subjects carrying 3-8 variant alleles demonstrated a significantly lower SSHL risk than subjects carrying 0-2 variant alleles. Consistent with our results, Li *et al* also found the combined genotypes with 3-8 variant alleles were associated with a decreased risk of NIHL

compared with those with 0-2 variant alleles (15). In regards to alcohol consumption, when the combined genotype consisted of 3-8 variant alleles, the risk of SSHL was lower compared with in subjects carrying 0-2 variant alleles. No association was found between the combined genotype frequency and age, gender and smoking status. Bibulosity is one of the potential risk factors of SSHL (31). Additionally, alcohol consumption can psychologically and physiologically interact with hearing loss (32).

The limitations of the present study are worth mentioning. The accumulation of retrospective data was not under control of the researchers analyzing the data, leading to inevitable bias. In addition, other types of gene should be taken into consideration when analyzing the genetic causes of SSHL.

Taken together, the *GRHL2* genetic polymorphisms, rs611419 and rs10955255, may confer protection against SSHL and reduce the risk of SSHL. The combination of the genotypes rs611419, rs10955255 and rs6989650 in the *GRHL2* gene is associated with a reduced risk of SSHL with more variant alleles. The present study provides fundamental genetic data for the *GRHL2* gene and demonstrates its association with SSHL, and thus the polymorphisms could be potential genetic biomarkers for investigating the mechanisms underlying SSHL.

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