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New SNP variants of *MARVELD2* (*DFNB49*) associated with non-syndromic hearing loss in Chinese population^{*,#}

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Non-syndromic hearing loss (NSHL) is a common defect in humans. Variants of *MARVELD2* at the *DFNB49* locus have been shown to cause bilateral, moderate to profound NSHL. However, the role of *MARVELD2* in NSHL susceptibility in the Chinese population has not been studied. Here we conducted a case-control study in an eastern Chinese population to profile the spectrum and frequency of *MARVELD2* variants, as well as the association of *MARVELD2* gene variants with NSHL. Our results showed that variants identified in the Chinese population are significantly different from those reported in Slovak, Hungarian, and Czech Roma, as well as Pakistani families. We identified 11 variants in a cohort of 283 NSHL cases. Through Sanger sequencing and bioinformatics analysis, we found that c.730G>A variant has detrimental effects in the eastern Chinese population, and may have relatively high correlation with NSHL pathogenicity.

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NSHL is a common human sensory defect (Smith et al., 2005; Morton and Nance, 2006). Up to now, more than 50 genes with autosomal recessive inheritance and 30 genes with autosomal dominant inheritance have been reported to be associated with NSHL (Dror and Avraham, 2009, 2010; Schraders et al., 2012). *MARVELD2*, which encodes tricellulin, is located on chromosome 5q13.2 and linked to the *DFNB49* locus (Ramzan et al., 2005; Riazuddin et al., 2006; Mašindová et al., 2015). In the human inner ear, there are many fluid-filled compartments of different ionic compositions. The strict compartmentalization plays a significant role in hearing. Tricellulin, together with other tight junction proteins, functions as a seal control lateral ion diffusion to ensure normal hearing (Sterkers et al., 1988; Kitajiri et al., 2004). Thus, it is easy to understand that variants in *MARVELD2* may cause hearing loss to varying degrees (Chishti et al., 2008). Up to now, seven single nucleotide polymorphism (SNP) variants of *MARVELD2* have been reported to cause human NSHL, including c.1183-1G>A, c.1331+1G>A, c.1331+2T>C, c.1331+2delITGAG, c.1498C>T, c.1543delA, and p.C395-Q501del (Riazuddin et al., 2006; Chishti et al., 2008; Babanejad et al., 2012; Šafka Brožková et al., 2012; Mašindová et al., 2015; Nayak et al., 2015). However, the mutational spectrum and frequency of *MARVELD2* in the Chinese NSHL population are still poorly understood. In the present study, a systematic mutational screening of *MARVELD2* was performed in a Chinese NSHL cohort.

A total of 283 genetically unrelated NSHL Chinese subjects were recruited. Informed consent, blood samples, and clinical evaluations were obtained from all participants under protocols approved by the

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Ethics Committee of both Zhejiang University and Wenzhou Medical University, China.

Genomic DNA was isolated from peripheral blood as detailed previously (Zheng et al., 2015). Nine pairs of primers were used to amplify the coding sequence of *MARVELD2* (Table S1). Polymerase chain reaction (PCR) products were identified by agarose gel electrophoresis, and the purified fragments were then analyzed by direct sequencing. Subsequently, bidirectional sequencing results were compared with a reference sequence from GenBank (NM_001244734).

A filter was created to analyze candidate variants, which consists of five rounds of screening, including allele frequency, amino acid conservation, amino acid substitution, detrimental prediction, and protein domain and structure analysis. The protein three dimensional (3D) structure and function were predicted by iterative threading assembly refinement (I-TASSER) (Yang et al., 2015).

All the statistical analyses were carried out by SPSS software (Version 21.0, SPSS Inc., Chicago, IL, USA). The chi-square test or Fisher's exact test was performed to evaluate the detrimental effect of all variants detected in the affected subjects, and *P*-value of <0.05 was regarded as statistically significant.

In order to study the mutation spectrum of *MARVELD2* in the Chinese population, we recruited 283 NSHL subjects from Zhejiang Province in China. This population consisted of 163 males and 120 females, including 191 profound, 47 severe, 37 moderate, and 8 mild hearing loss. Their ages of onset ranged from congenital to 60 years, with an average of 6.8 years.

DNA fragments spanning the entire coding region of *MARVELD2* were amplified by PCR. As shown in Table 1, 11 variants localized in exons were identified and all are heterozygous in affected subjects. Except for c.1499G>A, all variants are in exon 2 (Fig. 1a) which is the largest exon in tricellulin and includes the MARVEL domain.

In order to comprehensively analyze these variants, a sophisticated filter was created. The cut-down thresholds of frequency are less than 0.05 in both population groups (1000 Genomes, as well as the In-House Database provided by BGI China that is composed of 252 unaffected Chinese subjects). Next, we filtered out neutral or quiet mutations. The changes of the amino acid sequence of the expressed protein

may not necessarily affect protein structure or function. For example, the replaced amino acid may have very similar chemical properties to the amino acid of the original allele, in which case, the protein may still function normally. In this study, the c.230C>T and c.1109C>T variants caused amino acid change from Pro to Leu at position 77 and from Ala to Val at position 370, respectively. All these amino acids have the non-polar aliphatic side chain. Thus these variants were filtered out (Chasman and Adams, 2001; Teng et al., 2008). In addition, c.127G>A, c.1499G>A, c.1109C>T, c.98C>T, and c.592G>A did not pass the screen of bioinformatic tools (scale-invariant feature transform (SIFT) and PolyPhen-2) (Ng and Henikoff, 2003; Adzhubei et al., 2013). Finally, three variants c.772G>A, c.730G>A, and c.1006C>T passed the filter (Fig. 1b), and could be potential pathogenic variants responsible for NSHL. In addition, only c.730G>A had the *P*-value <0.05 among the three putative pathogenic variants filtered by our criteria when calculated by Fisher's exact test. For further confirmation, we used I-TASSER to predict the structure and function of *MARVELD2* (Yang and Zhang, 2015; Wang et al., 2017). As shown in Fig. 1c, c.730G>A causes a substitution of 244th glycine to arginine, which is predicted as a ligand binding site. This may lead to an abnormal interactive function of *MARVELD2* and eventually cause NSHL.

MARVELD2 encodes tricellulin, which is an important component of tricellular tight junctions and is involved in the formation of tricellular contacts (Ramzan et al., 2005; Riazuddin et al., 2006). In tight junctions, tricellulin minimizes permeability to macromolecules but not to ions, and this minimization is a significant factor in normal hearing (Krug et al., 2009). Raleigh et al. (2010) showed that MARVEL, occludin, and tricellulin have distinct but overlapping functions at the tight junction. Oda et al. (2014) reported that tricellulin also regulates F-actin organization through Cdc42 during cell-cell junction formation. It had been reported that variants in *MARVELD2* cause bilateral, moderate to profound NSHL (Riazuddin et al., 2006; Chishti et al., 2008). A gene-targeted knock-in (TricR497X/R497X) mouse was generated by Nayak et al. (2015), which aimed to mimic the pathology of a human *MARVELD2* variant. The results showed that deafness appears to be caused either by an increase in K⁺ ion concentration around the basolateral surfaces

Table 1 Variants in *MARVELD2* gene in 283 Chinese hearing-impaired subjects

Variant	Amino acid substitution	Amino acid property change	Exon	Detrimental effect (SIFT)	Detrimental effect (PolyPhen)	Conservation (%)	Allele frequency in 283 affected subjects (%)	Allele frequency in 252 controls (%)	<i>P</i> -value ^b (Fisher's exact test)	Allele frequency in ExAC (%)	
										East Asian	Total
*c.730G>A ^a	p.G244R	Non-polar aliphatic to alkaline	2/7	Damaging	Probably damaging	100.00	1.06	0	0.032		
*c.772G>A	p.V258M	Non-polar aliphatic to polar neutral	2/7	Damaging	Probably damaging	100.00	0.18	0	1.000	0	0
*c.1006C>T	p.R336W	Alkaline to aromatic	2/7	Damaging	Probably damaging	100.00	0.18	0	1.000	0	0
c.98C>T	p.T33I	Polar to non-polar	2/7	Tolerated	Benign	81.16	34.81	50.42	0	48.62	49.97
c.127G>A	p.A43T	Non-polar aliphatic to polar neutral	2/7	Damaging	Benign	85.19	0.18	0	1.000	0.01	0
c.130G>A	p.D44N	Acidic to polar neutral	2/7	Damaging	Probably damaging	88.89	0.18	0.42	0.604	0.12	0.01
c.230C>T	p.P77L	Non-polar aliphatic to non-polar aliphatic	2/7	Damaging	Probably damaging	100.00	0.18	0	1.000		
c.592G>A	p.V198M	Non-polar aliphatic to polar neutral	2/7	Tolerated	Probably damaging	85.88	0.53	0.85	0.713	0.24	0.02
c.949C>G	p.R317G	Alkaline to non-polar aliphatic	2/7	Damaging	Probably damaging	100.00	0.35	0.42	1.000	0.05	0
c.1109C>T	p.A370V	Non-polar aliphatic to non-polar aliphatic	2/7	Tolerated	Benign	85.19	0.18	0	1.000	0.07	0
c.1499G>A	p.R500Q	Alkaline to polar neutral	5/7	Tolerated	Benign	62.96	0.35	0	0.501	0.13	0.01

^a Variants that passed the filter perfectly were denoted by asterisks. ^b Statistical analysis of the variants detected in both controls and affected subjects was carried out by chi-square test; *P*-value of <0.05 was considered statistically significant. SIFT: scale-invariant feature transform; ExAC: the Exome Aggregation Consortium

Fig. 1 Schematic diagram of human tricellulin protein and the variants

(a) The proximate locations of 11 selected sense variants are shown; almost all these variants are in exon 2, only c.1499G>A in exon 5. Representative sequencing chromatograms of three variants which passed the filter are listed. (b) Protein structure of human tricellulin. MARVEL domains are shown in purple, and the occludin-ELL domain is in yellow. Red residues indicate the positions of mutations which pass the filter. (c) 3D structure of human tricellulin was predicted using the I-TASSER software. Protein domains are shown in grey, and ligands in green. Red spheres indicate the positions of mutations which pass the filter (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

or by an increase in small molecules such as adenosine triphosphate (ATP) around the hair bundle, leading to cellular dysfunction and degeneration (Higashi et al., 2013). Recently, Nayak et al. (2015) reported that *MARVELD2* variants are responsible for about 1.5% of NSHL in Pakistani families.

In this study, we analyzed *MARVELD2* variants in the Chinese population and identified an SNP variation (c.730G>A) which may have a correlation

with NSHL pathogenicity in that population. The c.730G>A variant showed a significantly higher frequency in the patient population (1.06%) than in controls (0.00%). In addition, c.730G>A caused a substitution for glycine to arginine, which may result in severe change of structure and function in the ligand binding domain and eventually lead to NSHL. Thus, our results indicated c.730G>A as a pathogenic variant of NSHL pathogenicity.

Table 2 Summary of all the pathogenic variants in *MARVELD2*

Mutation	Amino acids substitution	Origin of family	Reference
c.1183-1G>A		Pakistani	Riazuddin et al., 2006
c.1331+1G>A		Pakistani	Chishti et al., 2008
c.1331+2T>C		Pakistani, Czech Roma, Slovak Roma, Hungarian Roma	Riazuddin et al., 2006; Šafka Brožková et al., 2012; Mašindová et al., 2015
c.1331+2delTGAG		Pakistani	Riazuddin et al., 2006
c.1498C>T	p.R500X	Pakistani	Riazuddin et al., 2006
c.1543delA	p.K517RfsX16	Iranian	Babanejad et al., 2012
Exons 4–5 deletion	p.C395-Q501del	Pakistani	Nayak et al., 2015
c.1006C>T	p.R336W	Chinese	This report
c.730G>A	p.G244R	Chinese	This report
c.772G>A	p.V258M	Chinese	This report
c.949C>G	p.R317G	Chinese	This report

Up to now, seven SNP variants of *MARVELD2* causing NSHL have been reported in families of Pakistan, Slovak, Hungarian, and Czech Roma origins (Riazuddin et al., 2006; Chishti et al., 2008; Babanejad et al., 2012; Šafka Brožková et al., 2012; Mašindová et al., 2015; Nayak et al., 2015), as shown in Table 2. However, the *MARVELD2* variants identified in this study were not found in any of the previously reported studies. Our work profiles the spectrum and frequency of *MARVELD2* variants in the Chinese population, and this expands the *MARVELD2* variants pool. In addition, the study increases knowledge on *MARVELD2* variants causing NSHL, and this could be important for clinical diagnosis.

Contributors

Ye CHEN and Min-xin GUAN designed the project. Jing ZHENG and Wen-fang MENG performed data analysis and wrote the paper. Chao-fan ZHANG collected the data and wrote the paper. Han-qing LIU, Juan YAO, and Hui WANG carried out data processing.

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Compliance with ethics guidelines

Jing ZHENG, Wen-fang MENG, Chao-fan ZHANG, Han-qing LIU, Juan YAO, Hui WANG, Ye CHEN, and Min-xin GUAN declared that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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List of electronic supplementary materials

Table S1 Primers used in PCR

中文概要

题目：中国人群中非综合征耳聋相关 *MARVELD2* (*DFNB49*)基因新单核苷酸多态性位点分析

目的：探究 *MARVELD2* 在中国非综合征耳聋 (NSHL) 人群中的突变频谱和突变频率。

创新点：发现 *MARVELD2* 突变频谱具有明显种族特异性。中国 NSHL 人群中的突变位点及频率不同于已报道的其他人群，并首次筛选到新致聋候选突变 *MARVELD2* c.730G>A。本研究有助于进一步阐释 *MARVELD2* 在 NSHL 中的作用。

方法：收集 283 例 NSHL 患者外周血，提取基因组 DNA，涉及 9 对引物覆盖 *MARVELD2* 基因编码区，经聚合酶链反应 (PCR) 扩增后 Sanger 测序。测序结果与参考序列比对，获得的 *MARVELD2* 变异位点通过正常人群频率比较、氨基酸保守性分析、氨基酸性质分析、SIFT 和 PolyPhen 有害性预测及蛋白结构功能预测分析等进一步筛选得到耳聋候选突变位点。

结论：中国 NSHL 人群的 *MARVELD2* 突变位点与巴基斯坦人群，以及斯洛伐克、匈牙利和捷克罗马人群不同，具有明显的种族特异性。本研究在 283 个 NSHL 病例中共鉴定了 11 个变异位点。其中，c.730G>A 突变可能影响 *MARVELD2* 蛋白的正常功能，与 NSHL 致病有较高的相关性，是一个候选致聋突变。

关键词：*MARVELD2*；非综合征耳聋 (NSHL)；单核苷酸多态性位点