Identification of a novel MYO6 mutation associated with autosomal dominant non-syndromic hearing loss in a Chinese family by whole-exome sequencing

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Autosomal dominant non-syndromic hearing loss (ADNSHL) is characterized by postlingual progressive onset. Due to its high genetic heterogeneity, it is difficult to perform a molecular diagnosis for most patients with ADNSHL. In our study, whole-exome sequencing (WES) was used to screen pathogenic gene candidates by analyzing genomic DNA samples from a large Chinese family (JSNY-067), including the proband and her father, who suffered from non-syndromic hearing loss. The pathogenicity of candidate nonsynonymous variants in ADNSHL genes was evaluated by co-segregation analysis in family members by direct PCR and Sanger sequencing. Furthermore, multiple in silico analyses (SIFT, Polyphen2, PROVEAN and MutationTaster) and molecular dynamics simulation were used to assess the potential pathogenicity of the candidate mutations. We identified a novel causative mutation, c.622A>G in MYO6 (DFNA22), that resulted in a p.K208E substitution. This mutation co-segregated with the hearing loss phenotype in extended family members, and was predicted to be pathogenic by SIFT, PolyPhen2, PROVEAN and MutationTaster. Furthermore, molecular dynamics simulation analysis revealed that the p.K208E substitution had a limited influence on the whole protein structure and stability, but that it could affect the locations of the sidechains of nearby hydrophilic residues, which in turn resulted in the sidechains of Asn186 and Glu190 being exposed more frequently at the surface of the protein. WES has thus been shown to be a useful molecular diagnostic tool in screening uncommon gene mutations associated with hereditary hearing loss.

Key words: Whole-exome sequencing, MYO6, hearing loss, mutation, molecular dynamics simulation

INTRODUCTION

Hearing loss (HL) is one of the most common sensorineural disorders in humans, and affects over 277 million people worldwide. Depending on the diagnostic criteria, one out of 1000 children is born with a prelingual HL that can have a significant impact on normal speech and language skills (Sommen et al., 2017). The causes of HL include environmental factors, genetic factors, or a combination of both. Nevertheless, genetic factors are now regarded as the leading cause of HL in both developed and developing countries. It is estimated that approximately 70% of hereditary hearing loss (HHL) is non-syndromic (NSHL), wherein hearing impairment is the only feature observed, while the remaining 30% is classified as syndromic (Friedman and Griffith, 2003). Autosomal recessive NSHL (ARNSHL) is typically prelingual and is present in approximately 80% of HL patients, whereas the autosomal dominant types (ADNSHL) are mostly characterized by postlingual progressive onset, and they account for 20% of cases (Stelma and Bhutta, 2014).

To date, at least 100 genes have been implicated in NSHL, including 36 genes for ADNSHL (http://hereditaryhearingloss.org, accessed September 2017). Molecular diagnosis has become an important tool for investigating HHL etiology, especially for ADNSHL (Fang et al., 2017). The traditional method for ADNSHL diag-
nosis relies on the establishment of linkage to known DFNA loci in large pedigrees, followed by Sanger sequencing of candidate genes. This approach is not adaptable to phenotype misclassification, phenocopies caused by non-genetic causes, or the presence of locus heterogeneity within the same pedigree (Birkenhager et al., 2009). Additionally, this approach is labor-intensive and cost-ineffective for a disorder as heterogeneous as HL. An array-based method has also been developed, but this technique is relatively expensive and limited to certain gene mutations (Han et al., 2017). HHL is a disorder with a high genetic heterogeneity, and it is often difficult to identify the candidate causative genes via the above approaches.

Today, whole-exome sequencing (WES), which enables the simultaneous screening of multiple HL genes in multiple samples, is considered the most efficient and reliable tool for investigating the complexity of HHL. Gene panels are useful when multiple genes are involved in a particular disorder, or when there is an extensive phenotypic overlap between different disorders. Panels are also more cost-effective, and results can be obtained more rapidly than by the traditional gene-by-gene approach (Sommen et al., 2016).

The \( MYO6 \) gene is known to be responsible for both ADNSHL (DFNA22) and ARNSHL (DFNB37). It encodes unconventional myosin VI, which is necessary for the structural integrity and proper functioning of inner ear hair cells, including endocytosis, ion channel regulation, anchoring of stereocilia and vesicle movement (Oonk et al., 2013). Here, we used WES to investigate the genetic causes of ADNSHL in a large Chinese family (JSNY-067). Briefly, we identified a novel causative mutation, c.622A>G, in \( MYO6 \) (DFNA22), resulting in a p.K208E substitution, which was absent from 500 ethnically unrelated healthy individuals and 300 sporadic deafness cases. To elucidate the molecular etiology of HHL associated with c.622A>G in \( MYO6 \), we conducted an \textit{in silico} analysis, including homology modeling and molecular dynamics simulation (MDS), to analyze the probable loss-of-function mechanisms of myosin VI mutants associated with the deafness phenotype.

**MATERIALS AND METHODS**

**Patients and clinical evaluation** A large Chinese family (Fig. 1A) with ADNSHL spanning six generations was identified at the otology outpatient service in the Nanjing Children’s Hospital, Nanjing Medical University (China). All participating family members were evaluated through otological examination and audiological evaluations, including pure-tone audiometry, immittance, auditory brainstem response and distortion product otoacoustic emissions. Detailed medical histories were also collected, including degree of hearing loss, age of onset, symmetry of hearing impairment, use of hearing aids, noise exposure and other relevant clinical syndromic manifestations.

This study was approved by the Ethical Committee of Nanjing Medical University for Human Studies, and all participants read and signed an informed consent form.

**Molecular investigations** Genomic DNA was extracted from subjects’ blood using a DNA Extraction kit (Qiagen, Venlo, Limburg, the Netherlands) following the manufacturer’s instructions. All cases of deafness were pre-tested for nine hot mutations in four common deafness-causal genes found in the Chinese population (\( GJB2, SLC26A4, GJB3 \) and \( MT-RNR1 \)) using a Deafness Gene Mutation Detection Array Kit (Capital Bio Corporation, Beijing, China) as previously described (Wei et al., 2014).

**Exome capture and massive parallel sequencing** Approximately 3 \( \mu \)g of genomic DNA for each selected individual (unaffected: III-13; affected: V-3 and IV-3 (the proband and her father, respectively)) was extracted and purified from peripheral blood leukocytes using a QIAamp DNA blood kit (Qiagen). DNA was sheared into fragments of 150–250 bp (Covaris, Woburn, MA, USA) and purified using a MinElute PCR purification kit (Qiagen). The quality of fragmentation and purification was assessed by the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The DNA fragments were then end-repaired, adenylated, and ligated to NEBNext adaptors (NEB, Ipswich, MA, USA) for library preparation. A TruSeq DNA LT/HT Sample Prep Kit and a TruSeq Exome Enrichment Kit were used to enrich exomes. Equal amounts of library samples were pooled and then hybridized to the customized capture array, including exons, splicing sites and immediate flanking intron sequences. The whole exome library of each patient was sequenced on two lanes of the Illumina Genome Analyzer IIx using 71-bp paired-end reads. Raw image data were analyzed using Illumina CASAVA pipeline v1.7 to extract sequencing reads. Reads that aligned to exon regions were collected for mutation identification and subsequent analysis. Samtools (Ramirez-Gonzalez et al., 2012) mpileup was used for variant calling and identifying SNPs and indels. ANNOVAR (Wang et al., 2010) was used for gene annotation.

**Sanger sequencing** DNA samples from all available family members were subjected to direct PCR and Sanger sequencing to detect potential mutations in causative genes, especially in \( MYO6 \), which co-segregated with the disease phenotype in this family. PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI 3700XL Genetic Analyzer. The primers for the \( MYO6 \) sequencing were \( MYO6 \)-Forward:
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5'-TGTCCCGCCCATGTTAATAT-3'; and MYO6-Reverse: 5'-TGCACCGTACCTCAACACAG-3'.

In silico analysis SIFT (Kumar et al., 2009), Polyphen2 (Adzhubei et al., 2010), PROVEAN (Choi and Chan, 2015) and MutationTaster (Schwarz et al., 2010) were used to determine possible protein structure changes that might affect the phenotype. Clustal X1.83 (Aiyar, 2000) and PhyloP software (Liu et al., 2013) were used to compare the human wild-type MYO6 protein sequence with orthologs from Pan troglodytes, Mus musculus, Physeter catodon, Sus scrofa, Macaca fascicularis, Camelus bactrianus, Myotis lucifugus, Rousettus aegyptiacus, Bos taurus, Ailuropoda melanoleuca, Lipotes vexillifer, Octodon degus, Gallus gallus and Xenopus tropicalis, and to examine evolutionary conservation. The amino acid sequences were obtained from http://www.ensembl.org/.

Fig. 1. Pedigree and audiograms of the family JSNY-067. (A) Pedigree of Chinese family JSNY-067 with dominant progressive hearing loss. Open symbols represent unaffected individuals; filled black symbols denote affected individuals. The arrow indicates the proband. (B) Audiograms of six affected subjects. The severity of hearing loss and prevalence involvement appear to be correlated with the individual's age (yo: years old). Hearing loss ranged from mild to profound.
Homology modeling and molecular dynamics analysis

To examine the possible functional impacts of the MYO6 mutation (p.K208E) on the deafness phenotype, we further performed molecular dynamics simulation (MDS) for wild-type and mutant myosin VI protein. The initial structures of human myosin VI and its mutant p.K208E were homology-modeled from the template structure of 2BKH.A (Ménétrey et al., 2005). The PDB file 2BKH records the complex structure of the nucleotide-free motor domain-insert 2 of Sus scrofa myosin VI (chain A) with Drosophila melanogaster calmodulin (chain B). Residues 2-815 of human myosin VI were highly consistent with the template (799/814), but the remaining residues lacked suitable templates. Since the structures of residues 2-3, 356-360, 623-639 and 813-815 in the template were missing, only the 4-355, 361-622 and 640-812 parts of human myosin VI were modeled. Considering that the mutation point is far away from the missing parts of the protein, the modeled structures were used to investigate structural influences of the p.K208E mutation.

Due to the high similarity between the target sequence and the template, the modeled wild-type myosin VI and its p.K208E mutant were almost identical to the template structure. To further investigate the structural influences of the mutation, MDS was performed using Amber 12.0 (Case et al., 2015) and Ambertools 13.0 (University of California, San Francisco, USA). The ff14SB force fields (Maier et al., 2015) were used for protein residues, TIP3P water boxes were used to model the solvent environment, and Cl− ions were added to keep the system electrically neutral. Before dynamics simulation, 10,000 steps of energy minimization were performed with restraints on protein, followed by 10,000 steps of energy minimization without restraint, to remove possible collisions between atoms in the initial system. Next, the system was heated from 0 K to 300.0 K in 100 ps, and equilibrated to 1.0 bar in the following 5 ns. During the MDS, SHAKE constraints were added to all bonds involving hydrogen, and the time step was set to 2 fs. The Langevin thermostat (with the collision frequency set to 2.0 ps⁻¹) and Monte Carlo barostat (with the relaxation time set to 2.0 ps) were used to maintain the temperature and pressure. Other parameters were set to the program defaults. Finally, the equilibrated system was subjected to MDS for 100 ns at 300.0 K and 1.0 bar, and the atom coordinates were recorded every 10 ps to obtain the dynamics trajectory. For both the wild-type and p.K208E mutant, three independent simulations were performed, and the trajectories were analyzed.

RESULTS

Clinical investigation

As shown in Fig. 1A, the pedigree of family JSNJ-067 with postlingual ADNSHL included 14 affected individuals in six generations; audiograms were obtained from six affected family members. Except for their hearing impairment, all patients had a normal otorhinolaryngology status and no history of major neurological disorders. The hearing impairment was sensorineural, bilateral and progressive in all participating family members. At onset, the hearing impairment was detected at the middle and high frequencies, progressively degrading to a profound hearing impairment involving all frequencies. Furthermore, the hearing impairment was first noticeable at school age. Nevertheless, audiological examinations were delayed until hearing aids were required in adulthood. The progressive feature of the hearing impairment, which could also be indirectly deduced from PTA examinations performed at different ages, was reported by all participating family members (Fig. 1B, Table 1). Additionally, all individuals had a sensorineural HL, typically with a steeply sloping and flat audiogram. The severity of sensorineural HL was associated with age. There were no vestibular or retrocochlear dysfunctions, and no retinal diseases, in any of the participating family members.

Genetic analysis

To systematically search for deafness candidate genes, we performed whole-exome sequencing of samples from one unaffected (III-13) and two affected

| Patient | Age (years old) | Onset | Hearing impairment | Vestibular function | Tinnitus | Acoustic impedance admittance measurements | History of ototoxic drugs | History of environmental noise |
|---------|----------------|-------|-------------------|--------------------|-----------|------------------------------------------|---------------------------|-----------------------------|
| II-5    | 81             | Third decade | Progressive, severe | Normal | No | Normal | No | No |
| III-7   | 60             | Third decade | Progressive, profound | Normal | No | Normal | No | No |
| III-10  | 58             | Third decade | Progressive, moderate to profound | Normal | No | Normal | No | No |
| III-13  | 54             | –     | Normal | Normal | No | Normal | No | No |
| IV-3    | 38             | Second decade | Progressive, moderate | Normal | No | Normal | No | No |
| V-3     | 14             | First decade | Progressive, mild to moderate | Normal | No | Normal | No | No |
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(IV-3 and V-3) individuals from the JSNY-067 family. An average of 4.78 billion bases of high-quality sequence was generated per individual, with at least 80-fold average sequencing depth and 98% coverage of the target region, which satisfied the requirements for calling single-nucleotide polymorphisms (SNPs) and indels. The sequencing data were aligned to the NCBI human reference genome and compared with dbSNP138 (http://www.genome.ucsc.edu/egi-bin/hgGateway), the 1000 Genomes Project (http://www.1000genomes.org/), the HapMap database (http://www.hapmap.org) and the YH database (http://yh.genomics.org.cn).

An average of 6,778 SNPs and 819 indels were identified in the two patients after comparison with the above SNP databases, including splice acceptor and donor site mutations and coding indels (Supplementary Table S1, S2). These variants were then prioritized for further evaluation using three filtering criteria: (1) variants within the allele frequency cutoff (less than 0.01 in the dbSNP138, HapMap, 1000 Genomes and local datasets); (2) variants found in all the affected individuals but absent in the unaffected ones. (3) Nonsynonymous heterozygous variations meeting these criteria were selected for further analysis. A total of 189 variants were screened against SNP databases. Among them, 18 variants (including 12 nonsynonymous SNPs, splice acceptor and

Fig. 2. Mutation analysis of MYO6 in the Chinese family JSNY-067. (A) DNA sequences of heterozygous missense c.622A>G mutation and the wild-type control in exon 8. (B) Diagrammatic structure of myosin VI protein consisting of motor, IQ and coiled-coil domains. The p.K208E mutation maps in the motor head domain; conservation analysis indicated that the Lys residue at 208 in myosin VI protein is conserved in multiple vertebrate species.
donor sites, and six indels) were predicted to have a functional impact on the gene, and subjected to cosegregation analysis by direct PCR and Sanger sequencing among the pedigree samples. A missense variant, c.622A>G, p.K208E in exon 8 of the MYO6 gene, was detected to be co-segregated with the disease (Fig. 2A). This novel MYO6 mutation was exclusively identified in all 13 available affected patients, while no mutation was found in the 21 unaffected family members, or in 500 ethnically unrelated controls and 300 sporadic deafness cases.

**In silico analysis** To determine the potential effects of the p.K208E missense mutation on MYO6 function, we further performed in silico analyses. This mutation was predicted to be “Deleterious”, “Probably Damaging”, “Deleterious” and “Disease-causing” by SIFT, Polyphen2, PROVEAN and MutationTaster, respectively (Table 2). The conservation analysis indicated that the Lys residue at position 208 in the MYO6 protein is conserved across multiple vertebrate species (Fig. 2B). This finding indicated that this novel mutation may be the cause of the observed HL in this Chinese family.

**Homology modeling and MDS analysis** The root-mean-square deviation (RMSD) values of the protein backbone in the trajectories against the initial structure were first calculated to evaluate the whole protein structure stability (Fig. 3A). Due to the inherent high flexibility of the 397–405 loop and the lack of a subunit near the 774–812 helix of the C tail of the protein, these two parts were not included in the RMSD calculations. The RMSD plots showed that the RMSD values of both the wild-type and the p.K208E mutant were nearly stable during 40–100 ns in all trajectories, and the values were less than 2.5 Å. These data indicated that the whole protein structure of both the wild-type and the mutant was stable during the MDS. Therefore, the p.K208E mutation did not have a significant influence on the whole protein structure.

We next calculated the averaged residue root-mean-square fluctuation (RMSF) values during 40–60 ns, 60–80 ns and 80–100 ns of the three trajectories to study the residue fluctuations of the wild-type and mutant (Fig. 3B). The results showed that the residue RMSFs are almost identical (with differences less than 0.5 Å) between the wild-type and the mutant, suggesting that the p.K208E mutation had a limited effect on the residue fluctuations of the protein.

In the initial structure of the wild-type, the hydrophilic sidechain of Lys208 was located near the hydrophilic sidechain of Asn186, and the positively charged amino group may have electrostatically attracted the negatively charged carboxyl group of Asp456. When Lys mutated to Glu, the distance between the sidechain of residue 208 to the sidechain of Asn186 increased, and the negatively charged carboxyl group of Glu may have led to an electrostatic repulsion against Asp456. We therefore analyzed the distance between the CG atom of Asn186 and the CG atom of Asp456 during the last 60 ns of the trajectories. The result showed that the sidechain distance between Asn186 and Asp456 increased in the p.K208E mutant (Fig. 3C).

According to the representative structures of the wild-type and mutant obtained from clustering, the p.K208E mutant has more obvious influences on the positions of nearby hydrophilic residues than of hydrophobic residues. The amino group of Lys208 in wild-type attracted the carboxyl groups of Glu190 and Asp456, while the carboxyl group of Glu208 in the mutant repulsed the sidechains of Glu190 and Asp456. Since Asp456 is buried inside the protein, and has relatively strong inflexibility, this electrostatic repulsion had a limited influence on the sidechain position of Asp456, and resulted in position changes of the sidechain of Glu190 and of the mutated residue itself. From the representative structures, outward movement of the sidechain of Asn186 can also be observed. This change coincided with the increase in distance between the sidechains of Asn186 and Asp456 in the p.K208E mutant, as discussed above (Fig. 3D, 3E).

**DISCUSSION**

WES has recently been introduced as a powerful method for identifying causative gene mutations in Mendelian disorders. Compared with Sanger sequencing, WES is cost-effective when a large number of genes are screened (Hu et al., 2016). In the current study, we performed WES and successfully identified a novel MYO6 mutation.

| Gene       | DFN locus | Nucleotide change | Variation type | Amino acid change | SIFTa | Polyphen2 | PROVEANb | Mutation Taster | Allele frequency in controls | Novel or HGMD |
|------------|-----------|-------------------|----------------|-------------------|------|-----------|----------|------------------|----------------------------|---------------|
| MYO6       | 6q14.1    | c.622A>G          | Missense       | p.K208E           | 0.00 | Probably Damaging (0.995) | Disease-causing | 0.00             | Novel                       |               |

aScore ranges from 0 (deleterious) to 1 (neutral), with cut-off score set at 0.05. bNegative and positive scores indicate deleterious and neutral, respectively, with cut-off score set at −2.5. Variants with a score equal to or below −2.5 are considered “deleterious”; those above −2.5 are considered “neutral”.

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**Table 2. A novel variant in the proband of family JSNY-067**
Fig. 3. Molecular dynamics analysis of wild-type and K208E mutant myosin VI. (A) RMSD values of the protein backbone in the trajectories against the initial structure, which were used to evaluate the whole protein structure stability. (B) Averaged residue RMSF values during 40–60 ns, 60–80 ns, and 80–100 ns of the three trajectories, which were used to study the residue fluctuations of the wild-type and mutant. (C) Distance between the CG atom of Asn186 and the CG atom of Asp456 during the last 60 ns of the trajectories. (D) Statistics of the distance between the CG atom of Asn186 and the CG atom of Asp456 in wild-type and p.K208E mutant myosin VI. (E) Structural differences between wild-type and p.K208E mutant myosin VI.
MYO6 studies, hearing loss caused by not reported in the family medical history. In previous hypertrophic cardiomyopathy (Mohiddin et al., 2004) was involvement was excluded in all affected individuals, and deterioration (data not shown). Vestibular and visual tical analyses in this study failed to show significant thresholds were reported to be progressive, but statistics showed down-sloping audiograms and the hearing VI could result in progressive NSHL without vestibular ADNSHL in the JSNY-067 family. The mutant myosin MYO6 tion in exposed more frequently at the surface of the protein, resulted in the sidechains of Asn186 and Glu190 being conservation analysis indicated that the Lys208 residue in myosin VI is conserved in multiple vertebrate species. According to the results of MDS, the p.K208E mutation had little influence on the whole protein structure and stability, but affected the locations of the side-chains of nearby hydrophilic residues. This mutation resulted in the sidechains of Asn186 and Glu190 being exposed more frequently at the surface of the protein, which suggested that the c.622A>G (p.K208E) mutation in MYO6 could be pathogenic and responsible for ADNSHL in the JSNY-067 family. The mutant myosin VI could result in progressive NSHL without vestibular dysfunction in this family. The affected family members showed down-sloping audiograms and the hearing thresholds were reported to be progressive, but statistical analyses in this study failed to show significant deterioration (data not shown). Vestibular and visual involvement was excluded in all affected individuals, and hypertrophic cardiomyopathy (Mohiddin et al., 2004) was not reported in the family medical history. In previous studies, hearing loss caused by MYO6 has been identified as progressive (Topakal et al., 2010), although limited clinical details were available. In the present study, the proband and her father had bilateral, symmetrical, moderate sensorineural HL affecting high frequencies to the greatest degree, resulting in a down-sloping audiometric configuration. The onset was in the early postlingual period (10–20 years old), followed by steady and gradual progression. A history of progressive HL affecting several generations within the family implied an autosomal dominant inheritance pattern. The onset of sensorineural HL was similar in other affected family members (5–35 years old). The young subjects had sensorineural HL affecting only middle and high frequencies. In contrast, adults had hearing loss affecting all frequencies to varying degrees.

CONCLUSION

We identified a novel missense mutation of MYO6 in a Chinese family with ADNSHL, and conducted a preliminary analysis of the potential molecular pathogenesis of a myosin VI deafness mutation (p.K208E). This novel mutation expands the mutational spectrum of MYO6, and will contribute to a deeper understanding of the association between NSHL etiology and MYO6 mutations.

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