Mutation Research 790 (2016) 1–7

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Abnormal mRNA splicing but normal auditory brainstem response (ABR) in mice with the prestin (SLC26A5) IVS2-2A > G mutation

Jian Zhang[a], Ziyi Liu[a], Aoshuang Chang[b], Jie Fang[c], Yuqin Men[a], Yong Tian[c,d], Xiaomei Ouyang[e], Denise Yan[e], Aizhen Zhang[a], Xiaoyang Sun[a], Jie Tang[b], Xuezhong Liu[e,*], Jian Zuo[c,*], Jiangang Gao[a,*]

[a] School of Life Science and Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong University, Jinan 250100, China
[b] Department of Physiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China
[c] Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
[d] Laboratory of RNA Biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[e] Department of Otolaryngology, University of Miami, Miami, FL 33136, USA

ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 11 May 2016
Accepted 11 May 2016
Available online 12 May 2016

Keywords:
SLC26A5
IVS2-2A > G
Hearing loss
mRNA splicing
ABR

ABSTRACT

Prestin is critical to OHC somatic motility and hearing sensitivity in mammals. Several mutations of the human *SLC26A5* gene have been associated with deafness. However, whether the IVS2-2A > G mutation in the human *SLC26A5* gene causes deafness remains controversial. In this study, we created a mouse model in which the IVS2-2A > G mutation was introduced into the mouse *Slc26a5* gene by gene targeting. The homozygous *Slc26a5* mutant mice were viable and fertile and displayed normal hearing sensitivity by ABR threshold analysis. Whole-mount immunostaining using prestin antibody demonstrated that prestin was correctly targeted to the lateral wall of OHCs, and no obvious hair cell loss occurred in mutant mice. No significant difference in the amount of prestin protein was observed between mutants and controls using western blot analysis. In OHCs isolated from mutants, the NLC was also normal. However, we observed a splicing abnormality in the *Slc26a5* mRNA of the mutant mice. Eleven nucleotides were missing from the 5′ end of exon 3 in *Slc26a5* mRNA, but the normal ATG start codon in exon 3 was still detected. Thus, the IVS2-2A > G mutation in the *Slc26a5* gene is insufficient to cause hearing loss in mice.

1. Introduction

Prestin, which is critical to hearing sensitivity in mammals, is the OHC electromotility motor protein and encoded by the *SLC26A5* gene [1]. The inactivation of *Slc26a5* in mice resulted in the loss of OHC somatic motility *in vitro* and a 40–60 dB loss of cochlear sensitivity *in vivo* [2]. Different human *SLC26A5* mutations associated with hearing loss have been reported. Homozygosity for a 5′-UTR splice acceptor mutation (IVS2-2A > G) in exon 3 of the *SLC26A5* gene was identified in two Caucasian probands and purported to be responsible for the nonsyndromic deafness disorder DFNB61 [3]. By screening 47 Hungarian hearing impaired patients, another missense mutation (R150Q) in exon 6 of the human *SLC26A5* gene was found in one patient and his normal-hearing father, who were the heterozygous carriers [4]. Two Japanese sisters with moderate to severe hearing loss were found to have compound heterozygosity for two mutations, c.209G > A(p.W70X) and c.390A > C(p.R130S), in the human *SLC26A5* gene[5]. Hearing loss was associated with this compound heterozygous mutation, but not with individual heterozygous mutations. Because of the limited number of patients in these pedigrees, whether any of these *SLC26A5* mutations are true causes of hearing loss in these patients remains unclear.

In this study, we focused on the IVS2-2A > G mutation (HGVS: NM_206883.2:c.−53-2A > G; Genomic location: GRCh38 Chr7:103421569; dbSNP: rs116900495). For the IVS2-2A > G mutation, a high frequency of heterozygosity was observed only in Caucasian subjects, suggesting an association with a specific ethnic background [3]. However, in another study, the IVS2-2A > G transition was also found in other ethnic groups, and no significant

Abbreviations: OHC, outer hair cell; SLC26A5, solute carrier anion transporter family 26 member 5; ABR, auditory brainstem response; NLC, nonlinear capacitance.

* Corresponding authors.
E-mail addresses: xliu@med.miami.edu (X. Liu), jian.zuo@stjude.org (J. Zuo), jggao@sdu.edu.cn (J. Gao).

http://dx.doi.org/10.1016/j.mrfmmm.2016.05.004
0027-5107/

difference was observed in the allele frequency of the IVS2-2A > G nucleotide substitution between deaf patients and controls with normal hearing [6]. The expression of *SLC26A5* might be maintained in cells carrying the IVS2-2A > G mutation [6]. Other studies also supported the hypothesis that heterozygosity for the IVS2-2A > G mutation may not be sufficient to cause hearing loss [7,8]. Except for the two probands reported by Liu et al. [3], no subjects homozygous for this IVS2-2A > G mutation have been identified or studied in these reports. Therefore, whether the IVS2-2A > G mutation in *SLC26A5* is associated with deafness is still debatable.

Given that human *SLC26A5* and mouse *Slc26a5* genes are highly conserved, the creation of a mouse model with any of these human mutations may provide evidence for a causative genotype in humans. Mouse models of *Slc26a5* have been successful in providing mechanisms for prestin's role in cochlear amplification. The mouse model with the *Slc26a5*-C1 mutation involving three artificial point mutations (K233Q, K235Q, and R236Q) demonstrated that OHC somatic motility does not adjust the operating point of a stereocilia-based amplifier[9]. Another artificial *Slc26a5* mutation (V499G/Y501H) was used to diminish prestin's electromotile function without altering its membrane targeting, demonstrating that prestin-based electromotility is required for cochlear amplification [10]. However, such artificial *SLC26A5* mutations have not been associated with human deafness. Due to the inaccessibility of the cochlear tissues of deaf patients, the mechanisms for these reported mutations linked to human deafness remain unclear. Here we created a mouse model for the IVS2-2A > G mutation, which is the first deafness-linked mutation discovered in the human *SLC26A5* gene, and we examined the hearing sensitivity and the function of prestin in homozygous mutants, thus providing evidence regarding whether the IVS2-2A > G mutation causes deafness in humans.

2. Materials and methods

2.1. Generation of Slc26a5 knockin mice

We obtained three mouse *Slc26a5* bacterial artificial chromosome (BAC) clones, 399N2, 427N18 and 577A16 (cat. no. 96050, Research Genetics, Huntsville, AL), then we subcloned 2 overlapping *Slc26a5* genomic DNA fragments, 1B161 and P77, into Pbluescript. Plasmid 1B161 is a 9 kb BamHI fragment that contains exon3, and Plasmid P77 is a 3 KB PstI fragment which contains exon3 and exon4. The A–G change resembling the IVS2-2A > G mutation in human was introduced into the splicing region in front of exon 3 in the Plasmid 1B161 by using a QuickChange site-directed mutagenesis kit (cat. no. 200518, Stratagene, La Jolla, CA, USA). The targeting vector (PL452 as backbone) contained a 5 kb EcoRI-BamHI DNA fragment from the plasmid 1B161 as the long arm and a 2.2 kb BamHI-PstI DNA fragment from the plasmid P77 as the short arm. The targeting vector was transfected into 129S6 ES cells (cat. no. CMTI-1, Millipore, Billerica, MA, USA). Positive clones were screened by Southern blot analysis. Genomic DNA from ES cells was digested with SpeI and further hybridized with the external probe (exon 5). Targeted ES cell lines were selected and microinjected into the C57BL/6 blastocysts to generate chimera. The chimera were crossed with C57BL/6 mice to generate germline-transmitted mice, which carried both the mutation and the loxP-Neo-loxP cassette. The mice mentioned above were crossed with EIIa-Cre mice (ubiquitous Cre activity) to delete the neo gene and obtained heterozygous mutant mice (+/m). By intercrossing heterozygous mutant mice, the wild-type (+/+), +/m and homozygous mutant (m/m) mice were obtained. The mice were genotyped by PCR using primers (5′-ATGAGATCCACAGCCAGGAG-3′ and 5′-CTGTGGGAAGTGGCTTGACT-3′) flanking the remaining loxP site.

The mutant mice were confirmed by sequencing PCR products spanning the mutant site using primer pairs (5′-GTCACCAAGGACTTCCTCCA-3′ and 5′-GAAACTAGGACCCACGGTGA-3′). Genomic DNA was used as a template for the PCR.

All animal experimental protocols were approved by Ethics Committee of Shandong University. Animal management was performed strictly in accordance with the standards of the Animal Ethics Committee of Shandong University.

2.2. Whole-mount immunostaining

Whole-mount immunostaining was performed as described previously[11]. Mutant and wild-type mice at P30 were perfused intracardially with PBS followed by 4% paraformaldehyde in PBS. After post fixation at room temperature (RT), cochlea samples were dissected. The antibodies used were as follows: primary antibody, goat anti-prestin N-20 (1:300, cat. no. sc-22692, Santa Cruz Biotechnology, Santa Cruz, CA, USA); secondary antibody, rabbit anti-goat IgG (1:400, cat. no. ZF-0314, ZSGB-BIO, Beijing, China).

2.3. Western blot analysis

Four mutant (2 males and 2 females) and four wild-type (2 males and 2 females) mice at P30 were anesthetized and decapitated and their inner ears were dissected. Cochlear proteins were incubated in cell lysis buffer (10 mM Tris, pH = 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF) and extracted using a homogenizer. The western blotting protocol was also performed as described previously [11]. The primary antibodies used were goat anti-prestin N-20 (1:100, cat. no. sc-22692, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin rabbit antibodies (1:5000, cat. no. AP0731, Bioworld Technology, Minneapolis, MN, USA). The secondary antibodies used were donkey anti-goat IgG-HRP (1:2000, cat. no. sc-2020, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG-HRP (1:5000, cat. no. ZB-2301, ZSGB-BIO, Beijing, China). The protein bands were quantified using NIH image analysis software (ImageJ Version 1.48 V, National Institutes of Health, USA).

2.4. ABR analysis

ABRs were recorded and analyzed as described previously [12]. Nine mutant (5 males and 4 females) and eight wild-type (4 males and 4 females) mice at P30 were intraperitoneally anesthetized. Three electrodes were placed subcutaneously in the mice: one in the vertex, one underneath the left ear, and one on the back near the tail. ABR reflects the electrical response of the cochlear ganglion neurons and the nuclei of the central auditory pathway to sound stimulation. Their threshold assesses the cochlear sensitivity. Tone pips of 4 kHz, 8 kHz, 16 kHz, and 32 kHz were generated using a Tucker Davis Technologies (TDT) workstation (System III) running SigGen32 software (TDT).

2.5. NLC measurements

NLC measurements were performed on OHCs harvested from mice at 4–8 weeks as described [9]. Five mutant (3 males and 2 females) and seven wild-type (4 males and 3 females) mice were used. The organs of Corti were isolated from the cochleae and bathed in Leibovitz's L-15 medium (cat. no. 41300-039, Gibco, Life Technologies, Grand Island, NY, USA). After light enzymatic digestion for 5 min (1 mg/ml Collagenase IV, cat. no. C5138, Sigma, St. Louis, MO, USA) and gentle pipetting, the dissociated cells were transferred to small plastic chambers filled with enzyme-free L-15 (approximately 1.5 ml). The chamber containing the hair cells was then placed in the stage of a Nikon inverted microscope (Eclipse