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SHORT COMMUNICATION

Hair roots as an mRNA source for mutation analysis of Usher syndrome-causing genes

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mRNA is an important tool to study the effects of particular mutations on the mode of splicing and transcripts. However, it is often difficult to isolate mRNA because the organ or tissue in which the gene is expressed cannot be sampled. We previously identified two probable splicing mutations (c.6485+5G>A and c.8559-2A>G) during the mutation analysis of *USH2A* in Japanese Usher syndrome (USH) type 2 patients, but we could not observe their effects on splicing because the gene is expressed in only a few tissues/organs, and is not expressed in peripheral lymphocytes. In this study, we used hair roots as a source of mRNA of USH-causing genes, and successfully detected the expression of seven, except *USH1C* and *CLRN1*, of the nine USH-causing genes. We used RNA extracted from the hair roots of a patient who has both c.6485+5G>A and c.8559-2A>G mutations in *USH2A* in a compound heterozygous state to observe the effects of these mutations on transcripts. Reverse-transcription PCR analysis revealed that c.6485+5G>A and c.8559-2A>G inactivated splice donor and splice acceptor sites, respectively, and caused skipping of exons. Thus, RNA extracted from hair roots is a potential powerful and convenient tool for the mutation analysis of USH-causing genes.
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INTRODUCTION

To perform mutation analysis in the study of hereditary diseases, we generally used genomic DNA for the detection of mutations in exons and adjacent intronic regions of the gene of interest. Further, mRNA, if available, can also be used for the detection of mutations and for determining their effects on transcripts. However, except in the cases when the gene of interest is expressed in blood cells, it is difficult to isolate mRNA because the organ or tissue in which the gene of interest is expressed cannot be sampled.

We recently performed mutation analysis of *USH2A* gene for Japanese patients of Usher syndrome (USH) type 2, and identified 14 mutations, including 11 novel ones.[1] Of these mutations, two were splicing mutations, c.6485+5G>A and c.8559-2A>G in introns 33 and 42, respectively. We determined the pathogenicity of these mutations using supportive data, but could not examine their effect on pre-mRNA splicing because of the difficulty in obtaining *USH2A* mRNA. The expression of *USH2A* mRNA is restricted to a few tissues, including the retina and the cochlear, and is absent in peripheral lymphocytes.[2] Similarly, peripheral lymphocytes do not express mRNA of any other USH-causing genes, except *DFNB31*.[3]

Here, we attempted to use hair roots as a source of USH-causing gene mRNA. We successfully detected the mRNA expression of most USH-causing genes and analyzed the effect of the above-mentioned *USH2A* mutations on pre-mRNA splicing. This is the first report on the mRNA expression of USH-causing genes in hair roots.

MATERIALS AND METHODS

Collection of hair roots

At least 30 hair root samples were collected from the scalp of normal Japanese individuals and a USH type 2 patient. The patient had c.6485+5G>A and c.8559-2A>G mutations in *USH2A* in a compound heterozygous state (see the patient C152 in a previous report[1]). The institutional review board of Hamamatsu University School of Medicine approved this study, and written informed consent was obtained from all participants before enrollment.

Reverse-transcription PCR of USH-causing genes

Total RNA was extracted from the hair roots using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Next, 2 μg of total RNA was reverse transcribed with oligo(dT) primers by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) for all nine known USH-causing genes was amplified using specially designed PCR primers (Table 1). The PCR mixtures (total volume, 20 μl) contained 2 μg cDNA, 1.0 M betaine (Wako, Osaka, Japan), 1.5 mM $MgSO_4$, 0.3 μM each primer and 0.4 U KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The amplification conditions were as follows: denaturation at 94 °C for

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Table 1 Nucleotide primers designed for PCR and sequencing of Usher syndrome-causing genes

Template	*Primer sequences (5′ to 3′)*	*Exon*	*Annealing temperature (°C)*[a]	*Product length (bp)*
MYO7A cDNA	F: TGAGATTGGGGCAGGAGTTCGACG	2	68	428
	R: GATGATGCAGCACTGGTCTCGGCT	4		
USH1C cDNA	F: AGTGGCCCGAGAATTCCGGCATAA	1	64	359
	R: CTGCCTGACCGCCTTTGATGAGGT	4		
CDH23 cDNA	F: GGTCGGCTTTGCCCTTCCACTCTT	11	64	449
	R: GTCCCGTGTCCTTGTCCAGCGAGA	14		
PCDH15 cDNA	F: TGCCAAACACTCGTGATTGCCGTC	8	64	330
	R: GACCGGCAAAGGCAGGAAGAGGAT	11		
USH1G cDNA	F: CCCACTCTCTGGGCTGCCTACCAT	1	68	443
	R: GTGAGGCTGGAGAAGCTGAGGGTGT	2		
USH2A cDNA	F: TAACTGCTTGCACTTTGGCTGGCT	31	64	613
	R: GTTAGGGCCTCACTGGCCTCACTC	35		
USH2A cDNA	F: GTGGTGACAGTGCTGGAACCCGAT	41	64	563
	R: ACAGTCACTTCTCGGCTCGGTGTAA	44		
GPR98 cDNA	F: ACTCACCTTTTGGCTTGGTGGGCT	53	64	533
	R: AAAGCTTCCAGCCAGCCGGACTAC	56		
DFNB31 cDNA	F: CTGCGCGTCAACGACAAATCCCTG	1	64	371
	R: CCTGGGTCCACGCCAGTGATGTAA	3		
CLRN1 cDNA	F: GCAATCCCAGTGAGCATCCACGTC	2	64	368
	R: GGGAACTGAAATCCAGCAAGTCGT	3		

Abbreviations: F, forward; R, reverse.
[a]The amplification conditions were as follows: denaturation at 94 °C for 2 min; followed by 40 cycles of treatment at 98 °C for 10 s, 64 or 68 °C for 30 s (see this column), and 68 °C for 1 min; and final extension at 68 °C for 5 min.

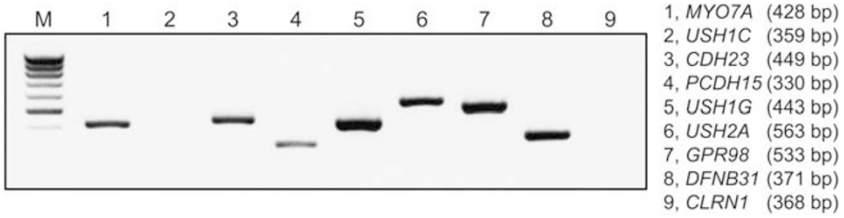

Figure 1 RT-PCR analysis of USH-causing genes. mRNA expression of all USH-causing genes, except *USH1C* and *CLRN1*, was detected in normal control hair roots. PCR was performed using 2 μg cDNA (total volume, 20 μl) with 40 cycles.

2 min; followed by 40 cycles of treatment at 98 °C for 10 s, 64 or 68 °C for 30 s (described in Table 1) and 68 °C for 2 min; and final extension at 68 °C for 5 min.

RESULTS

Detection of mRNA of USH-causing genes in hair roots

Total RNA was prepared from the scalp hair root samples obtained from normal individuals. Reverse-transcription PCR (RT-PCR) analysis revealed the mRNA expression of all USH-causing genes, except *USH1C* and *CLRN1*, in hair roots (Figure 1).

Detection of the splicing abnormality caused by *USH2A* splicing mutations

We next attempted to detect the splicing abnormality caused by the compound heterozygous mutations c.6485+5G>A and c.8559-2A>G in *USH2A*. Total RNA was prepared from the hair root samples obtained from the patient, and RT-PCR was performed using primers to amplify the cDNA between exons 31 and 35. Agarose gel electrophoresis of the RT-PCR products revealed two bands—a larger band corresponding to the normal sequence and a smaller band corresponding to the mutant sequence (Figure 2a). Sequence analysis of the mutants revealed that c.6485+5G>A causes skipping of exon 33 (160 bp) and presumably creates a premature stop codon in exon 34 through a frameshift. Similarly, RT-PCR performed using primers to amplify the cDNA between exons 41 and 44 revealed that c.8559-2A>G causes skipping of exon 43 (123 bp) (Figure 2b) and presumably induces a 41-amino-acid deletion. These results revealed that c.6485+5G>A and c.8559-2A>G inactivated splice donor and splice acceptor sites, respectively, and this finding confirmed the pathogenicity of these mutations.

DISCUSSION

RT-PCR analysis revealed the mRNA expression of seven of the nine USH-causing genes in hair roots. It has been reported that the mRNA of one USH-causing gene, *MYO7A* (causes USH type 1B), can be detected in the nasal epithelium;[4] however, obtaining *MYO7A* mRNA would necessitate invasive and painful tissue sampling methods. In contrast, collecting hair roots from the scalp is not an invasive procedure. Further, analysis of total RNA obtained from the hair roots of the patient with USH type 2 revealed that the two intronic mutations c.6485+5G>A and c.8559-2A>G inactivated a splice donor and splice acceptor sites, respectively, and both these mutations resulted in exon skipping. This is the first report to describe the RT-PCR analysis of *USH2A* mutations and show that the mutations close to the splice donor/acceptor sites cause splicing errors.

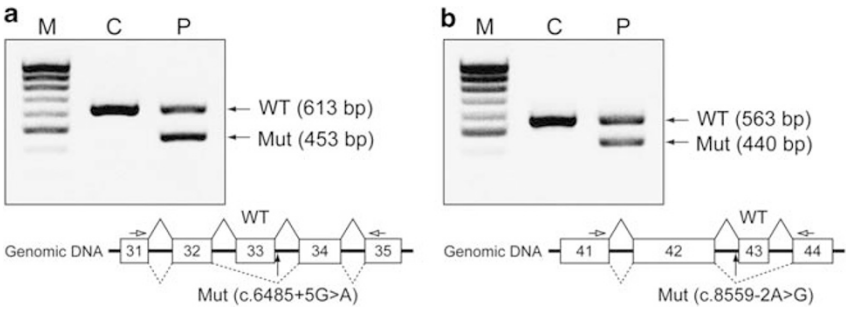

Figure 2 (**a**) Products of RT-PCR performed using primers to amplify *USH2A* cDNA between exons 31 and 35. The c.6485+5G>A mutation caused skipping of exon 33 (160 bp) and was presumed to create a premature stop codon in exon 34 through a frameshift. (**b**) Products of RT-PCR performed using primers to amplify *USH2A* cDNA between exons 41 and 44. The c.8559-2A>G mutation caused skipping of exon 43 (123 bp) and was presumed to create a 41-amino-acid deletion. Boxes with a number represent the exons. The solid and dotted lines that connect exons show the manner of splicing in the wild type and mutant, respectively. The distance between exons does not indicate the actual intron sizes. The open arrows indicate the PCR primers, and the closed arrows indicate mutations in introns. M, molecular marker (100 bp ladder); C, control; P, patient; WT, wild type; Mut, mutant.

Generally, mRNA is very useful for mutation analysis, especially in the case of coding-sequence mutations in large multi-exon genes, splicing mutations and regulatory-region mutations that affect the expression levels. Of these, the use of mRNA to determine the effect of a mutation on splicing as we revealed in this report is the most important advantage because we still cannot accurately predict splicing changes from DNA sequence alterations, especially if the alterations occur at a distance from splicing donor/acceptor sites[5] or within exonic splicing enhancers.[6]

Thus, mRNA extracted from hair roots is a potentially powerful and convenient tool for mutation analysis in USH-causing genes. Further, it is also reasonable to hypothesize that the mRNA of genes that cause deafness can be detected in hair roots, and this may facilitate easier and more accurate mutation analysis.

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