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Molecular characterization of pathogenic *OTOA* gene conversions in hearing loss patients

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Abstract

Bi-allelic loss-of-function variants of *OTOA* are a well-known cause of moderate-to-severe hearing loss. Whereas non-allelic homologous recombination-mediated deletions of the gene are well known, gene conversions to pseudogene *OTOAP1* have been reported in the literature but never fully described nor their pathogenicity assessed. Here, we report two unrelated patients with moderate hearing-loss, who were compound heterozygotes for a converted allele and a deletion of *OTOA*. The conversions were initially detected through sequencing depths anomalies at the *OTOA* locus after exome sequencing, then confirmed with long range PCRs. Both conversions lead to loss-of-function by introducing a premature stop codon in exon 22 (p.Glu787*). Using genomic alignments and long read nanopore sequencing, we found that the two probands carry stretches of converted DNA of widely different lengths (at least 9kbp and around 900bp respectively).

Keywords

Deafness; Pathogenic gene conversion; Long read sequencing; Otoancorin

Mendelian genetic defects constitute a leading cause of non-syndromic hearing loss, one of the most common sensorineural defects in humans (Nance, 2003). Although dominant and recessive phenotypes linked to *GJB2*/*GJB6* are a frequent etiology (Yokota et al., 2019),

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more than 150 genes have been linked with syndromic or non-syndromic deafness (Azaiez et al., 2018; Sloan-Heggen et al., 2016). Consequently, genetic diagnosis has moved from screening of the connexin locus to interrogating simultaneously thousands of exons of the human genome, to reach an overall diagnostic rate of around 40% (Azaiez et al., 2018; Shearer & Smith, 2015).

STRC and *OTOA* bi-allelic loss-of-function are well-known contributors of autosomal recessive hearing loss in humans (Mehta et al., 2016; Vona et al., 2015; Zazo Seco et al., 2017). Both *STRC* and *OTOA* reside in repetitive regions of the genome, leading to recurrent deletions or duplications (copy number variants, CNVs) in the general population, through non-allelic homologous recombination (Zhang, Gu, Hurles, & Lupski, 2009). These regions also encompass paralogous pseudogenes for both genes: *CKMT1A-STRCP1-CATSPER2P1* lie directly downstream of *CKTM1B-STRC-CASTPER2* on chromosome 15. The 16p12.1 band, where *OTOA* and *OTOAP1* are located, is a complex and wrongly oriented region in the GRCh37 and GRCh38 human assemblies (Antonacci et al., 2010). The region has been corrected with a fix patch on GRCh38 (Refseq ID NW_017852933.1), and the two loci are now predicted to be less than 300kbp apart and outwardly oriented.

Pathogenic and likely pathogenic frameshift, nonsense, missense and splicing variants have been deposited to ClinVar (Landrum et al., 2016) for *OTOA* and *STRC*. Using array Comparative Genomic Hybridization (aCGH), Hoppman et al. (2013) found an allele frequency of 1.09% for the *STRC-CATSPER2* deletion, over a cohort of more than 5000 patients. The gnomAD structural variant (SV) dataset v2.1 (Collins et al., 2020), calling CNVs using read depth from more than 10000 whole genome sequencing data, reports an allele frequency of 0.90% for the deletion of *STRC* specific exons. However, deletions of *OTOA* are apparently much rarer, with estimates ranging from less than 0.1% in gnomAD SV v2.1 to less than 0.2% in the Database of Genomic Variants (MacDonald, Ziman, Yuen, Feuk, & Scherer, 2014).

Gene conversion, defined as the replacement of a locus in the genome by a paralogous sequence, is a mechanism known to generate pathogenic alleles (Casola, Zekonyte, Phillips, Cooper, & Hahn, 2012; Chen, Cooper, Chuzhanova, Férec, & Patrinos, 2007). For instance, a pathogenic converted allele has been described for the *TMEM231* gene, causing autosomal recessive Joubert and Meckel-Gruber syndromes (Maglic et al., 2016; Watson et al., 2020). Other examples include a benign conversion of 88 to 351 nucleotides of *BRCA1* intron 2 (Tessereau et al., 2015) mediated by the same breakpoint sites leading to a 37kbp-long deletion of exon 1 and 2 of *BRCA1*.

In their cohort of 686 patients with hearing-loss, using high throughput sequencing data, Shearer et al. (2014) reported that CNVs in *STRC* were the most common of all CNVs found in deafness-related genes, followed by CNVs in *OTOA*. Interestingly, they highlighted the case of a causative homozygous gene conversion of *STRC*. The authors also mentioned 3 conversions of *OTOA*, which length was estimated between one to two unspecified exons. No pathogenic CNVs nor single nucleotide variants (SNV) were detected in trans in any of the 3 patients, and the pathogenicity of the conversion itself was not assessed. The conversions were detected by comparative analysis of sequencing depth in the paralogous

regions of the genes and pseudogenes, but without confirmation with another molecular approach. Moteki et al. (2016) also reported the case of a homozygous gene conversion of *STRC* in a cohort of 40 patients, identified by high throughput sequencing and confirmed by aCGH. More recently, Rajagopalan et al. (2020) identified two single exon gene conversions of *STRC*, initially identified as single exon deletions in exome sequencing CNVs calls, confirmed with long range PCR. In this work, we report two unrelated patients with hearing loss who both inherited a deleted allele and a converted allele of *OTOA*.

The first proband is a young female child (case 1) whose parents are cousins and of Kurdish origin. The proband's neonatal auditory screening (otoacoustic emissions) failed for both ears. After four repeated failures she was referred for a full hearing evaluation. At 4 months of age, a moderate sensorineural hearing loss was highlighted by brainstem evoked response audiometry (Supp. Figure 1), with a hearing threshold estimated at 50 dB. Behavioural audiometry confirmed the threshold. At two years of age, a tonal audiogram (Supp. Figure 2) repeatedly showed a moderate hearing loss and the proband was referred for genetic investigations. The proband has no dysmorphic features. There was no familial history of hearing problems and the couple has a 18-months older daughter with normal hearing.

Exome sequencing performed on the DNA extracted from the blood (Supp. Methods) of the first proband did not reveal any pathogenic or likely pathogenic SNV in our panel of 187 genes. However, the algorithm for CNV detection (Supp. Methods) showed z-scores compatible with bi-allelic *OTOA* variants (Fig. 1A and 1B), consisting of a heterozygous deletion spanning at least 110kb from exon 2 of *METTL9* (NM_016025.5) to exon 22 of *OTOA* (NM_144672.4) and a smaller variant centered around exon 22 (z-score compatible with a homozygous deletion of exon 22). The MLPA analysis confirmed the large 110kb deletion and showed it was inherited from the mother (Supp. Methods and Supp. Table S1).

The resolution of the paternal allele was hindered by the fact that the region spanning intron 20 to exon 29 of *OTOA* displays very high sequence identity with exons 1 to 9 of the *OTOAP1* pseudogene (NR_003676.3) and therefore lacks MLPA probes. Upon visual inspection of the reads aligning to exon 22 of *OTOA*, we hypothesized the presence on the paternal chromosome of either a small deletion of exon 22 of *OTOA* or of a conversion of the wild-type sequence of *OTOA* by *OTOAP1*.

A 13kb PCR product specific to the paternally inherited *OTOA* allele was obtained using the proband's DNA and primer #1 and #2; primer #1 being specific to *OTOA* and primer #2 to both *OTOA* and *OTOAP1* (Supp. Methods and Fig. 1C). This PCR product was then Sanger sequenced using a set of internal primers listed in Supp. Table S2.

Taken together the sequencing results showed that the paternal *OTOA* allele was not carrying a deletion of exon 22 but underwent pseudogene-mediated gene conversion. This conversion event introduced several sequence variants contributed by *OTOAP1* into *OTOA* including among others a pathogenic premature stop codon (p.Glu787*) (Fig. 1E).

To determine the starting point of the conversion event, we sequenced the 13kbp amplicon using a MinION nanopore sequencer (Supp. Methods) and assessed known differences between *OTOA* and *OTOAP1* reference genomic sequences (Fig. 2).

The first *OTOAP1* specific nucleotide was found approximately 350 bp upstream of *OTOA* exon 21. All expected downstream sequence differences corresponded to *OTOAP1* sequences, at the hemizygous state, up to the end of the amplicon at exon 23. This fine mapping analysis allowed us to map the start of the conversion to a 334bp window, and to estimate its length to at least 9kbp. We reported the conversion with the following HGVS nomenclature: NC_000016.10:g.(21730155_21730489)_(21739516_?)con(22546282_22546616)_(22555638_?). Finally, we designed a 3kb PCR product around the mapped conversion start window with primers #1 and #7 (Fig. 1C and Supp. Table S2) and confirmed the presence of the converted allele in the proband and her father, the conversion being absent in the mother (Fig. 1D).

Following the characterization of the converted allele in our proband, we identified one ClinVar submission (accession SCV000966854.1) reporting the stop codon mutation contributed by *OTOAP1*, p.Glu787*, in trans with a deletion, identified in a patient with hearing loss (case 2). Proband 2 is a Caucasian female child presenting with congenital mild-to-moderate sensorineural hearing loss with no family history. She was 3 months old when referred for genetic investigations. The *OTOA* deletion was initially detected by sequencing depth analysis after targeted hybridization capture and high throughput sequencing (Supp. Methods).

After obtaining DNA from this patient, we confirmed the deletion of *OTOA* on one chromosome using MLPA (Supp. Table S1) and performed the long range amplification and nanopore sequencing as described above. In contrast with the first proband, only 11 positions around exon 22 showed nucleotides specific to *OTOAP1*, including the three in the coding sequence (p.Thr785Pro, p.Glu787* and p.Tyr806Ser). This allowed us to estimate that the size of the conversion on this second allele was less than 900bp and to code the conversion with the following HGVS nomenclature: NC_000016.10:g.(21735999_21736311)_(21736714_21736886)con(22552122_225552435)_(22552837_22553008).

Gene conversion leading to pathogenic variants has already been described in a variety of human Mendelian diseases (Chen et al., 2007; Chen, Férec, & Cooper, 2010). They have been hypothesized to arise after a double-strand break where the damaged chromosome is repaired, after resection, using a non-allelic homologous region of either the sister chromatid or the homologous chromosome. However, it has been assumed that, in humans, conversion events longer than 3kbp are more likely occurring via a non-allelic homologous double recombination mechanism, because most well characterized gene conversion events typically replace regions of a few hundreds of nucleotides only (Chen et al., 2007). For instance, this mechanism has been proposed to be at the origin of the 12f2 deleted lineages of the human Y chromosome (Blanco et al., 2000). A formal distinction between the two mechanisms would require characterization of the by-product chromosome, which is not possible when investigating human alleles.

Given the lengths of the two conversions we described (more than 9kbp and less than 900 bp), we assume that the mechanisms at the origin of these two alleles are respectively non-allelic homologous double recombination and double strand break repair-mediated gene

conversion events between *OTOA* and *OTOAP1*. Comparison between *OTOA* and *OTOAP1* reference sequences from GRCh38 shows identity higher than 99% over more than 42 kbp of aligned nucleotides, which is sufficient to promote non-allelic homologous recombination between the two loci (Gu, Zhang, & Lupski, 2008).

Heterozygous conversion of one to two exons of *OTOA* was reported in 3 patients among a cohort of 686 patients with hearing loss (Shearer et al., 2014), without direct molecular confirmation. Estimating the allelic frequencies of the conversion in the population from exome sequencing or short-reads whole genome sequencing is non-trivial. It requires coverage depth comparison between regions of *OTOA* and *OTOAP1*. Using exome sequencing, only the exons showing sufficient divergence, allowing for unique mapping to either loci, can reliably be called as converted or not. The alignments of *OTOA* and *OTOAP1* exonic sequences from GRCh38 show differences only for exons 21, 22 and 29 and their 50 flanking nucleotides, with respectively 1, 7 and 1 changes. For the evaluation of pathogenicity, only the conversion of exon 22 appears relevant, as the change in exon 21 is synonymous and the one in exon 29 lies in the 3'UTR.

It is likely that polymorphisms in regions of segmental duplication in *OTOA* and *OTOAP1* are under evaluated due to the difficulty of genotyping non-unique sequences. Interestingly, the p.Thr785Pro, p.Glu787* and p.Tyr806Ser mutations of *OTOA* exon 22 are present in gnomAD v2.1.1 (Karczewski et al., 2020) as low frequency alleles, as well as low quality alleles for p.Thr785Pro and p.Glu787*. The corresponding opposite mutations on *OTOAP1* exon 2 are also present in this database. These observed substitutions could result from errors in the mapping algorithm, leading to sequencing reads being incorrectly assigned to their paralogous regions in the genome. The alternative hypothesis would be that recurrent conversions between the two loci create alleles that appear as a mixture of *OTOA* and *OTOAP1* wild-type specific changes. This hypothesis is consistent with the observation that gene conversions contribute to a significant part of the shared polymorphism between paralogous regions in the human genome (Dumont, 2015; Dumont & Eichler, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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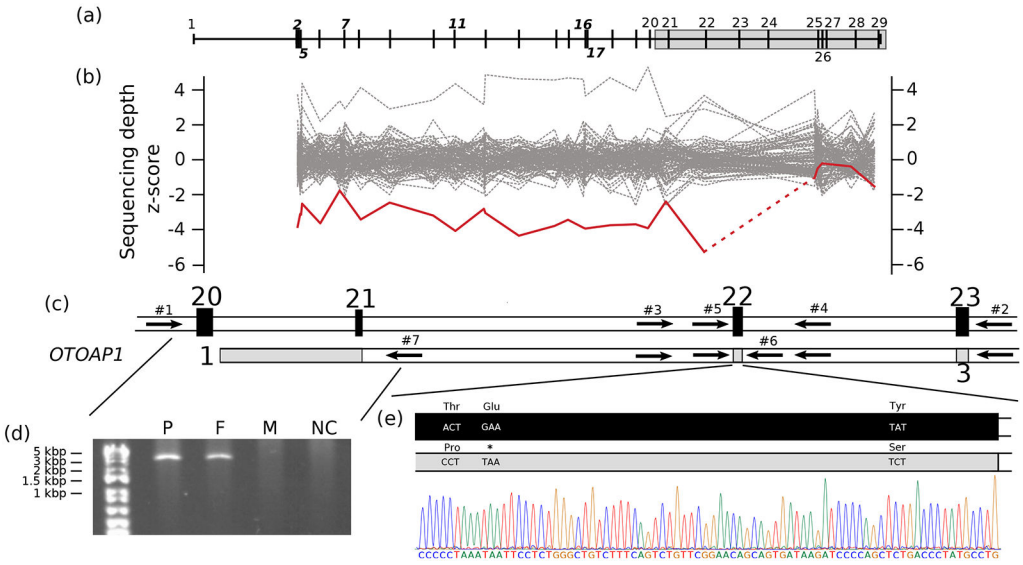

Figure 1.

Genomic investigations in case 1. (a) Schematic representation of the 29-exon *OTOA* gene. The first exon is noncoding. Italic bold exons (2, 5, 7, 11, 16, and 17): locations of *OTOA* MLPA probes. Gray rectangle: segmentally duplicated region of *OTOA* sharing high level of sequence identity with *OTOAP1*. The especially high level of identity between exons 23 and 29 of *OTOA* and exons 3-9 of *OTOAP1* make the CNVs detection unreliable. (b) NGS sequencing depth in Case 1 (red line) compared with controls (grey dashed lines) indicating a heterozygous deletion. The broken red line indicates the region of *OTOA* where paralog sequence homology hampers mapping quality and limits sequencing depth, preventing z-score calculation. (c) Schematic view of the alignment between the reference sequences of *OTOA* exon 20-23 and *OTOAP1* exon 1-3 with regions targeted by our set of primers. Primer #1 is specific to *OTOA* whereas primers #6 and #7 are specific to *OTOAP1*. (d) Detection of the converted *OTOA* allele: Gel electrophoresis after amplification using primers #1 and #7 confirms the paternal inheritance of the conversion. P: proband, F: father, M: mother, NC: negative control. (e): Resolution of the converted *OTOA* allele: A 13kb polymerase chain reaction product (primer #1 & #2) was obtained from the proband converted allele and exon 22 was sequenced using primer #5 & #6. As expected all three *OTOAP1*-specific substitutions are found in a hemizygous state. The exon is partially shown, with its Sanger-sequence, bottom track.

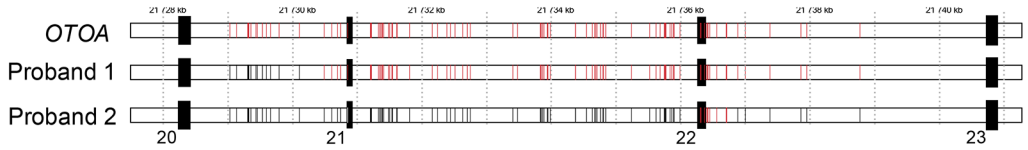

Figure 2.

Fine mapping of the *OTOA* conversion using nanopore sequencing of the 13kbp amplicon (Primer#1 & #2) in both probands. Red marks on the *OTOA* locus represent every predicted difference between *OTOA* (NG_012973.2) and *OTOAP1* (NC_000016.10, coordinates 22545698-22576865) when aligning genomic sequences. Black marks indicate positions where the *OTOA* nucleotide was found in the nanopore sequencing mapping, whereas red marks indicate positions where the *OTOAP1* nucleotide was found. This analysis indicates that the conversion was at least 9 kbp in proband 1 but less than 1 kbp and restricted to exon 22 in proband 2.