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Comprehensive genetic testing with ethnic-specific filtering by allele frequency in a Japanese hearing-loss population

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Abstract

Recent advances in targeted genomic enrichment with massively parallel sequencing (TGE+MPS) have made comprehensive genetic testing for non-syndromic hearing loss (NSHL) possible. After excluding NSHL subjects with causative mutations in *GJB2* and the *MT-RNR1* (1555A>G) variant by Sanger sequencing, we completed TGE+MPS on 194 probands with presumed NSHL identified across Japan. We used both publicly available minor allele frequency (MAF) datasets and ethnic-specific MAF filtering against an in-house database of 200 normal-hearing Japanese controls. Ethnic-specific MAF filtering allowed us to re-categorize as common 203 variants otherwise annotated as rare or novel in non-Japanese ethnicities. This step minimizes false-positive results and improves the annotation of identified variants. Causative variants were identified in 27% of probands with solve rates of 35%, 35% and 19% for dominant, recessive and sporadic NSHL, respectively. Mutations in *MYO15A* and *CDH23* follow *GJB2* as the frequent causes of recessive NSHL; copy number variations in *STRC* are a major cause of mild-to-moderate NSHL. Ethnic-specific filtering by allele frequency is essential to optimize the interpretation of genetic data.

Keywords

Hearing loss; deafness; ethnicity; targeted genomic enrichment; massively parallel sequencing

INTRODUCTION

Hearing loss is the most common sensory disorder. As a congenital finding, it affects one in every 500 newborns, while its impact as a global problem is huge (1, 2) – the World Health Organization estimates that 360 million persons have significant hearing loss. It is genetically heterogeneous with over 89 genes causally implicated in autosomal dominant,

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Conflict of interest

All authors have declared no conflict of interests.

recessive, X-linked or mitochondrial non-syndromic hearing loss (NSHL - hearing loss in the absence of other phenotypic findings), although more than 140 loci have been mapped (Hereditary Hearing Loss Homepage, <http://hereditaryhearingloss.org/>).

Recent advances in targeted genomic enrichment with massively parallel sequencing (TGE +MPS) have revolutionized the diagnosis of genetic hearing loss and had a significant impact on the clinical evaluation of deaf/hard-of-hearing patients (3). In this study, the first of its kind in a Japanese population, we used TGE+MPS to screen a large number of persons with NSHL to identify the spectrum of genetic deafness in Japan. We illustrate the power of ethnic-specific MAF filtering to reduce the potential false-positive rate in the genetic diagnosis of NSHL (4).

SUBJECTS and METHODS

Subjects

One hundred ninety-four (194) Japanese subjects (114 females) from unrelated and non-consanguineous families were identified in 33 otolaryngology clinics in 28 prefectures across Japan. All subjects had presumed NSHL. For each proband, informed consent was obtained to participate in this study, which was approved by the human subjects ethical committee associated with each clinic. Clinical information and blood samples were obtained on all consenting affected and unaffected family members. Collected data included: 1) pure tone audiograms, behavioral audiometry or auditory brain stem responses (ABR); 2) medical history, including onset of hearing loss, progression and episodes of vertigo; and 3) temporal bone imaging (computed tomography and/or magnetic resonance) if these tests had been done. Probands with Sanger sequence identification of pathogenic mutations of *GJB2* or *MT-RNR1* (1555A>G) were excluded. Other exclusion criteria included: 1) apparent syndromic hearing loss; 2) possible environmental hearing loss (i.e., birth injury or ototoxic drugs); and 3) insufficient high-quality genomic DNA (gDNA). Hearing levels were classified based on the better hearing ear as: normal, < 20 dB; mild hearing loss, 21–40 dB; moderate hearing loss, 41–70 dB; severe hearing loss, 71–95 dB; and profound hearing loss, >95 dB.

Targeted Genomic Enrichment and Massively Parallel Sequencing

The TGE of all exons of all genes implicated in NSHL, including NSHL mimics, was completed as described, targeting 66 or 89 genes as part of the OtoSCOPE® v4 or v5 platforms, respectively (see Supporting Information, Table S1 for full list)(5). Libraries were prepared using a modification of the solution-based Agilent SureSelect target enrichment system (Agilent Technologies, Santa Clara, CA)(6). In brief, 3µg of gDNA was randomly fragmented to an average size of 250 bp (Covaris Acoustic Solubilizer; Covaris Inc., Woburn, MA), and adaptor ligated before the first amplification. Hybridization and capture with RNA baits was followed by a second amplification before pooling for sequencing. Minimal amplification was used – typically 8 cycles for the pre-hybridization PCR (range 8–10 cycles) using NEB Phusion HF Master Mix (New England BioLabs Inc, Ipswich, MA) and 14 cycles for the post-hybridization PCR (range 12–16 cycles) using Agilent Herculase II Fusion DNA Polymerase. All samples were barcoded and multiplexed before sequencing

on either an Illumina MiSeq or HiSeq system (Illumina Inc, San Diego, CA) in pools of 4–6 or 48, respectively, using 100-bp paired-end reads.

Bioinformatics Analysis

Data were analyzed using a local installation of the open-source Galaxy software (<http://galaxyproject.org>) and the following open-source tools: BWA (7) for read mapping, Picard for duplicate removal, GATK (8) for local re-alignment and variant calling and NGSRich (9) for enrichment statistics (5). We reported and annotated variants with custom software. Copy number variations (CNVs) were determined using an R script based on a read-depth approach (10) followed by expert curation of identified CNVs (5).

Variant Analysis

Six in-silico pathogenicity prediction algorithms were used to assess the possible impact of non-synonymous and non-splice variants, with a score of 1 given for each of the following tools that assigned a variant as pathogenic: PhyloP, SIFT, PolyPhen2, LRT, Mutation Taster, GERP. The maximum pathogenicity score (PS) was 6, and we considered those variants with a PS >4 as likely pathogenic (5). In addition, variants were evaluated in terms of mode of inheritance (i.e., recessive vs. dominant), phenotype (i.e., enlarged vestibular aqueduct, *SLC24A4*; delayed motor milestones, Usher syndrome type 1), and audioprofile using AudioGene (11).

We retained variants with minor allele frequency (MAF) ≥ 0.005 as reported in the 1000 Genome project (<http://www.1000genomes.org/>) and the Exome Variant Server (<http://evs.gs.washington.edu/EVS>). We also compared identified variants with our in-house database, termed the OtoSCOPE Control Database (OtoDB), which includes 1000 normal-hearing individuals from six populations sequenced on the same platform using a pooled method (4). The latter includes Japanese normal-hearing controls (described below). All pathogenic variants were confirmed by Sanger sequencing and segregation analysis using exon-specific custom primers.

Statistical Analysis of MAF

OtoDB includes six populations consisting of (1) 200 Ashkenazi Jews, (2) 160 Colombians, (3) 160 European Americans, (4) 180 Spanish, (5) 100 Turkish, and (6) 200 Japanese (all with normal hearing as documented by audiometry). For ethnic-specific MAF filtering, we used MAF data from 200 Japanese controls (OtoDB-JP). We used the chi-square test with Yate's correction to compare differences between identified variants in the Japanese NSHL patients and the 1,000 Genome project database and OtoDB (1,000 normal-hearing) for all candidate variants. We also completed a linear discriminant analysis to classify the variants as pathogenic or polymorphisms.

RESULTS

Sequencing Quality and Variant Identification

The 194 gDNA samples were prepared in three sets: Sets 1 and 2 were prepared manually while Set 3 was prepared robotically. The average per-sample total number of reads was

6,928,896, with 10X target coverage of 99.3% (Supporting Information, Table S2). By using a variant quality filter to identify high quality variants and a MAF filter to select rare variants, we reduced the number of plausible variants to 7 and 10 per sample for OtoSCOPE[®] v4 and OtoSCOPE[®] v5, respectively (Fig. 1a). The 1677 variants identified in the 194 probands were classified as novel (569), rare (574 MAF <0.5%) or common (534, MAF >0.5% and <2%) using MAF data from 1000 Genomes and the EVS (Fig. 1b). We then filtered using ethnic-specific MAF filtering based on 200 normal-hearing Japanese controls, thus reducing the number of variants categorized as novel (552 vs. 569) and rare (387 vs. 574), and increasing the number of variants categorized as common (738 vs. 534) (Fig. 1b). In the group of 552 novel variants were 58 candidate pathogenic variants validated by Sanger sequencing and segregation analysis. The average PS of these variants was 5.2, while the average PS of the remaining novel variants was 3.8.

P-values and MAF of identified variants

To clarify the efficiency of each MAF filtering step, we compared MAFs across the 1,000 Genome database, OtoDB and OtoDB-JP, calculating *p*-values for 619 candidate variants after removing variants with MAF >2% as reported in the 1000 Genome database and EVS (Figure 2). Linear discriminant analysis gives the proportion of variants for which discrimination between causative and rare polymorphisms failed. In the OtoDB-JP, all causative variants (black points) are below the discrimination boundary.

Diagnostic Rate and Clinical Presentations

TGE+MPS enabled us to identify the genetic cause of hearing loss in 35%, 35% and 19% of patients segregating dominant, recessive or sporadic NSHL, respectively. When classified by age-of-onset as congenital (<1 year), childhood, adulthood (> 18 years old) or unknown (detected at a school wellness check but previously unaware of hearing loss), diagnostic rates were 26–36% in each category with the exception of adulthood, in which the diagnostic rate was only 17% (3 of 17 probands) (Table 1).

Genetic Spectrum of Hearing Loss in the Japanese

Amongst probands segregating autosomal dominant NSHL, variants in *KCNQ4* (OMIM 600101) and *WFS1* (OMIM 600965) were identified in four families each, followed by *MYO6* (OMIM 606346) and *TECTA* (OMIM 601543). The most common causes of autosomal recessive NSHL were variants in *MYO15A* (OMIM 600316), *CDH23* (OMIM 601386) and *SLC26A4* (OMIM 600791). We also identified CNVs of the *STRC* (OMIM 603720) genetic region as an important cause of hearing loss (Table 2).

DISCUSSION

The value of genetic testing in NSHL

In this study, we used TGE+MPS to identify the genetic cause of NSHL in 52 of 194 (27%) Japanese patients in whom mutations in *GJB2* and *MT-RNR1* (1555A>G) had been excluded (12). Based on the 14% contribution that *GJB2* mutations are estimated to make to the NSHL genetic load in Japan (13), in the absence of exclusionary criteria, the diagnostic rate for TGE+MPS in a Japanese hearing-loss population would approximate 40%, which is

similar to results reported in an American population (5). When considered by inheritance pattern, the diagnostic rate was 35% for both autosomal dominant and recessive patterns of inheritance, but only 19% in the sporadic cohort. When the subgroup of sporadic patients with congenital NSHL was considered separately, the diagnostic rate was 27% (3 of 11) for mild-to-moderate NSHL and 23% (13 of 57) for severe-to-profound SNHL (Fig. 3).

The importance of ethnic-specific MAF filtering

We used general MAF filtering based on data from the 1,000 Genomes Project and EVS but purposefully *also* included a filtering step based on an in-house database (OtoDB) of MAFs for 200 Japanese with normal hearing. The importance of this step is illustrated by our identification of a *LOXHD1* (OMIM 613079) variant, c.1883G>A, p.Gly628Asp, which has a MAF of 0.09% in the 1,000 Genome and a PS of 4 of 6, but a MAF of 1.3% in the Japanese controls (OtoDB-JP). Another variant, *CDH23* c.1681T>G, p.Phe561Val has a MAF of 0.14% in the 1000 Genome and a PS of 4 of 6, but its MAF in the Japanese population is 2.45% (OtoDB-JP). In aggregate, ethnic-specific MAF filtering reduced our list of novel and rare variants from 1143 to 950, illustrating the power of this step and stressing the need for deeper and, therefore, richer databases of multiple ethnicities to identify low frequency variants specific to ancestry groups and often unique to specific populations (14). Statistical analysis by chi-square test showed that ethnic-specific MAF filtering was useful in removing non-causative variants, even when their MAF was low (Fig. 2). This step is essential to minimize false-positive results (15).

Genetic spectrum in a Japanese population

Results from our study suggest that in the Japanese population, mutations in *KCNQ4* are the most common cause of autosomal dominant NSHL, while *GJB2*, *MYO15A* and *CDH23* are the most frequent causes of autosomal recessive NSHL. In other studies of the Japanese population, *GJB2* mutations were found in exceptionally high numbers, followed by mutations in *SLC26A4*, *USH2A*, *GPR98*, or in *CDH23*, *SLC26A4*, *MYO15A* (16) (17). Comparisons across multiple populations are difficult due to variations in patient selection criteria and enrollment numbers, however mutations in *MYO15A* appear to be common in several areas of the world (18) (19).

Relevant to our results is the fact that the analysis pipeline we have developed incorporates CNV calling, an added step that is not routinely included in most studies. CNVs can be detected in any targeted gene, which is relevant to the genetic diagnosis of NSHL (20). We identified for the first time CNVs of *STRC* as a cause of autosomal recessive and sporadic NSHL in this population. Although our numbers are limited, our data suggest that CNVs of *STRC* are a major cause of mild-to-moderate hearing loss.

In conclusion, in ~40% of Japanese patients with hearing loss a genetic etiology can be identified using TGE+MPS. To optimize the performance of this technology, ethnic-specific filtering by allele frequency is essential. The analysis pipeline must also include tools to call CNVs. We believe that TGE+MPS should be offered after audiometry and prior to other tests as a cost-saving step in the diagnosis of hearing loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overview of the variant filtering strategy. **(a)** Number of variants in each filtering step. Q/D, QD scores (Phred-like quality score divided by depth); MAF, minor allele frequency; EVS, Exome Variant Server; dbSNP, Single Nucleotide Polymorphism Data base; OtoSCOPEdb, OtoSCOPE database of variants. **(b)** The total number of variants in 194 patients. The upper circle graph shows the distribution of novel, rare and common variants classified by 1,000 Genome, EVS-AA and EVS-EA. The lower circle graph shows the numbers filtered out by ethnicity-specific MAF filtering. Two hundred and three variants (17 novel, 186 rare) were categorized as common variants.

PS, pathogenicity score, using six in-silico pathogenicity prediction algorithms: PhyloP, SIFT, PolyPhen2, LRT, Mutation Taster, GERP. The maximum score was 6, and we considered those variants with a PS >4 as likely to be pathogenic.

(Large deletion in the *STRC* gene is not included in these graphs.)

Figure 2.

Scatter plot of the comparison between identified variants and each minor allele frequency (MAF) level by 1,000 Genome, 1,000 normal-hearing OtoSCOPE database and 200 normal-hearing Japanese OtoSCOPE database (used as an ethnic-specific MAF filtering) by the chi-square test. All circles are candidate variants and black points indicate causative variants. In the comparison with the 200 normal-hearing Japanese (OtoDB-JP), the majority of the candidate variants aggregate around the low p -value area (upper-left), whereas variants are dispersed over the high p -value area in the comparison with 1,000 Genome and OtoSCOPE database. In the OtoDB-JP, all causative variants are below the boundary of the linear discriminant analysis.

Figure 3.
Diagram of genetic testing for hearing loss and overall results of this study, classified by mode of inheritance, hearing level and onset of hearing loss.

Table 1

Diagnostic Rate and Clinical Presentation

		Diagnosis	Total (n)	Diagnostic rate
All Cases		52	194	27 %
	Dominant	18	51	35 %
	Recessive	14	40	35 %
	Sporadic	20	103	19 %
Age of Onset	Congenital-1 year	27	96	28 %
	Childhood	18	70	26 %
	Adult>18 years	3	17	17 %
	Unknown	4	11	36 %
Type of hearing loss	Mild-Moderate	25	95	26 %
	Severe-Profound	27	99	27 %
	Progressive	20	68	29 %
	Stable	27	103	26 %
	Unknown	5	23	22 %

Table 2

Diagnosis by Mode of Inheritance

Inheritance	n of patients	Genes	n of patients	% of all solved patients
AD	18	<i>KCNQ4</i>	4	7.5 %
		<i>WFS1</i>	4	7.5 %
		<i>MYO6</i>	3	5.7 %
		<i>TECTA</i>	2	3.8 %
		<i>TMC1</i>	2	3.8 %
		<i>MYH14, MYO7A, P2RX2</i>	1 each	1.9 %
AR	34	<i>MYO15A</i>	6	11.3 %
		<i>CDH23</i>	5	9.4 %
		<i>SLC26A4</i>	4	7.5 %
		<i>OTOF</i>	3	5.7 %
		<i>STRC</i>	3	5.7 %
		<i>LOXHD1, PCDH15 PTPRQ MT-RNR1 (961T>C)</i>	2 each	3.8 %
		<i>GPR98, MYO7A, TECTA, TMPRSS3</i>	1 each	1.9 %
X linked		<i>POU3F4</i>	1 each	1.9 %