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Hereditary hearing loss: a 96 gene targeted sequencing protocol reveals novel alleles in a series of Italian and Qatari patients

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ABSTRACT

Deafness is a really common disorder in humans. It can begin at any age with any degree of severity. Hereditary hearing loss is characterized by a vast genetic heterogeneity with more than 140 loci described in humans but only 65 genes so far identified. Families affected by hearing impairment would have real advantages from an early molecular diagnosis that is of primary relevance in genetic counseling. In this perspective, here we report a family-based approach employing Ion Torrent DNA sequencing technology to analyze coding and UTR regions of 96 genes related to hearing function and loss in a first series of 12 families coming from Italy and Qatar. Using this approach we were able to find the causative gene in 4 out of these 12 families (33%). In particular 5 novel alleles were identified in the following genes *LOXHD1*, *TMPRSS3*, *TECTA* and *MYO15A* already associated with hearing impairment. Our study confirms the usefulness of a targeted sequencing approach despite larger numbers are required for further validation and for defining a molecular epidemiology picture of hearing loss in these two countries.

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1. Introduction

Hearing loss is the most frequent sensory deficit affecting human beings. It is an extremely heterogeneous disease that can begin at any age with any degree of severity. Hereditary hearing loss (HHL) can be syndromic (SHL) (about 25%), in which deafness is accompanied by other signs and/or symptoms, and non-syndromic (NSHL) (about 75%), in which there are no additional abnormalities (Kemperman et al., 2002). Genetic transmission is autosomal recessive in 75–85% of cases (ARNSHL), autosomal dominant in 15–25% (ADNSHL), while X-linked or mitochondrial inheritance accounts for a small proportion of cases (1–2%) (Smith et al., in press). Based on the age of onset, HHL can also be described as pre-lingual or post-lingual (after and before speech development respectively). For most genes/mutations that segregate with a recessive pattern of inheritance, the associated phenotype is characterized by a pre-lingual non-progressive hearing loss whereas mutations segregating with dominant pattern typically lead to post-lingual progressive hearing loss.

Considering the complexity of the hearing mechanism, it should come as no surprise that many genes are involved in hearing function and loss. Together with the clinical heterogeneity, HHL is also characterized by vast genetic heterogeneity with more than 140 loci but only 65 genes associated with NSHL so far have been described (http://hereditaryhearingloss.org/). Moreover, high allele heterogeneity is also present with more than 1000 causing mutations identified (http://deafnessvariationdatabase.org) (Shearer et al., 2011; Van Camp and Smith, 2012). Known genes encode proteins with diverse functions such as transcription factors, cell adhesion molecules, and ion channels (Dror and Avraham, 2010). Among them *GJB2*, *GJB6* and A1555G mitochondrial mutations play a major role worldwide varying from population to population (Guan, 2011; Van Camp et al., 1997).

In Italy, roughly 60% of cases still need to be characterized at molecular level (Cama et al., 2009; Rabionet et al., 2000), while in Qatar a recent publication showed that *GJB2* accounts for less than 15% of cases and no mutations in *GJB6* or mitochondrial A1555G were reported (Khalifa Alkowari et al., 2012). Thus, to further increase our knowledge on the molecular bases of HHL and to provide a molecular diagnosis to patients from these countries we have developed a targeted sequencing

Abbreviations: HHL, hereditary hearing loss; SHL, syndromic hearing loss; NSHL, non-syndromic hearing loss; ARNSHL, autosomal recessive non-syndromic hearing loss; ADNSHL, autosomal dominant non-syndromic hearing loss; HLTSP, Hearing Loss Targeted Sequencing Panel; CCDS, Consensus Coding Sequence; UTR, untranslated region; INDELs, Small Insertions and Deletions; SNVs, Single Nucleotides Variations; VCF, Variant Call Format; ESP, NHLBI Exome Sequencing Project; QUAL, quality score; OAEs, Otoacustic Emissions; NMD, nonsense-mediated decay; WES, whole exome sequencing.

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protocol to simultaneously analyze 96 genes belonging to the following categories: 1) known HHL causing genes in humans, 2) known HHL causing genes in animal models, and 3) genes with a potential role in the inner ear development. Using this protocol we have screened 12 Italian and Qatari families affected by either ARNSHL or ADNSHL leading to the identification of 5 novel alleles in 4 genes (*LOXHD1*, *MYO15A*, *TMPRSS3* and *TECTA*) already known to be associated with hearing impairment.

2. Materials and methods

2.1. Patients

Five Italian families characterized by sensorineural bilateral HHL have been included in the study (three characterized by ADNSHL and two by ARNSHL). Additional seven Qatari families affected by ARNSHL have been investigated. All families were negative for the presence of vestibular signs and symptoms and none of them showed any syndromic features. Furthermore, diabetes, cardiovascular diseases, visual problems and neurological disorders were also excluded. All families included in the study were completely negative for the presence of mutations in *GJB2* and *GJB6* genes as well as for the presence of the A1555G mitochondrial mutation. At least two individuals per family have been analyzed by sequencing, both affected and healthy ones. All patients provided written informed consent form for both genetic counseling and molecular genetic testing prior to enrolment. Written informed consent was obtained from the next of kin on behalf of the minors/children involved in this study. The study was approved by the Institutional Review Board of Hamad Medical Corporation, Doha, Qatar and by the Institutional Review Board of IRCCS Burlo Garofolo, Trieste, Italy. All research was conducted according to the ethical standards as defined by the Helsinki Declaration.

2.2. Hearing Loss Targeted Sequencing Panel (HLTSP)

A Hearing Loss Targeted Sequencing Panel (HLTSP) including genes related to hearing loss and function was defined according to data obtained from scientific literature and from the most comprehensive public databases (http://hereditaryhearingloss.org, http://deafnessvariationdatabase.org/, http://www.informatics.jax.org/). The panel includes 96 genes as reported in Supplemental Table S1. Briefly, the panel contains 42 ARNSHL genes and 20 ADNSHL genes; 5 genes related to both dominant and recessive patterns of inheritance; 12 SHL genes (4 causing dominant syndromic forms and 8 recessive ones); 5 genes causing X-linked forms. Moreover, 7 genes related to hearing function and loss in mice models have been included as well as 5 genes whose protein localization has been detected in ear structures (i.e. cochlea or nervous hearing system) and/or which are known to be involved in inner ear or hair cell development and cochlear wiring.

A primer pool intended for DNA library construction through multiplex PCR was designed using Ion AmpliSeq™ Designer v1.2 (Life Technologies, CA, USA). A targeted region represents a coding region (CCDS), 5′UTR, 3′UTR and also 50 bp exons/introns boundaries of the 96 genes under investigation. The primer design ensures 92% overall targeted region coverage spanning through 411K bp.

2.3. Targeted and Sanger DNA sequencing

DNA libraries were constructed using Ion AmpliSeq Library Kit 2.0 and indexed using Ion Xpress Barcode Adapters Kit (Life Technologies, CA, USA) according to the manufacturer's protocols. Template Ion Sphere Particles were prepared using Ion PGM Template OT2 200 kit and a single end 200 base-read sequencing run was carried out using Ion PGM sequencing 200 kit v2 (Life Technologies, CA, USA) on Ion Personal Genome Machine System (Life Technologies, CA, USA). Four indexed patients' libraries were sequenced simultaneously on each Ion 318™ Chip. Sequencing data were analyzed in accord with the Ion Torrent Suite™ v3.6; Single Nucleotides Variations (SNVs) and Small Insertions and Deletions (INDELs) were collected into a standardized Variant Call Format (VCF) version 4.1 (Danecek et al., 2011). SNVs and INDELS were then annotated using ANNOVAR (Wang et al., 2010). The most likely disease-causing SNVs/INDELs were analyzed by direct Sanger sequencing on ABI PRISM 3130xl sequencer (Life Technologies, CA, USA), using ABI PRISM 3.1 Big Dye terminator chemistry (Life Technologies, CA, USA) accordingly to the manufacturer's instructions. Sanger sequencing was employed both to confirm targeted sequencing results and to perform segregation analysis.

2.4. Mutation pathogenicity assessment

A comparison between identified genetic variants and data reported in NCBI dbSNP build137 (http://www.ncbi.nlm.nih.gov/SNP/) as well as in 1000 Genomes Project (http://www.1000genomes.org/) and NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/) led to the exclusion of those genetic variants previously reported as polymorphism. The impact of missense mutations on the protein structure was tested using several in silico predictor tools such as Polyphen-2 (Adzhubei et al., 2013), MutationTaster (Schwarz et al., 2010), SIFT and LRT (Liu et al., 2013). Moreover, conservation of residues across species was evaluated by PhyloP algorithm (Pollard et al., 2010). In order to exclude variants not segregating within the analyzed family, SNVs/INDELs were filtered by VCFtools (http://vcftools.sourceforge.net/). The presence of mutated alleles was checked in 100 DNA samples of ethnically matched healthy controls.

3. Results

The HLTSP was employed to analyze 12 families affected by HHL and completely negative for mutations in *GJB2* and *GJB6* genes, as well as for the A1555G mitochondrial mutation.

A mean of 165 Mbp of raw sequence data was produced per individual. On average 95% of the targeted region was covered at least 20-fold, and 260-fold mean-depth total coverage was obtained. An average of 487 genetic variants (SNVs/INDELs) was called per subject; we filtered out SNVs/INDELs with quality score (QUAL) <20 as well as synonymous nucleotide substitutions. Moreover we excluded from any further analysis all the variants already reported in dbSNP as polymorphism with the following exclusion criteria: SNPs with MAF >0.03 in families with a recessive pattern of inheritance and all SNPs in families with a dominant pattern of inheritance. The overall filtering process led to an average of 17 residual SNVs/INDELs for each subject, all of them as candidate HHL causative mutations (Fig. 1). We then focused our attention on the potential HHL genetic variants segregating within each family starting from those predicted as disease causing by several in silico predictor tools.

Five novel alleles in 4 HHL genes (*LOXHD1*, *TMPRSS3*, *TECTA* and *MYO15A*) were identified, in four out of the 12 investigated families. Pedigrees of these four families are shown in Fig. 2; arrows indicate family members analyzed by HLTSP.

In Family 1, coming from Italy, two missense mutations (c.1019C>G and c.1291C>T) in *TMPRSS3* gene (NM_024022.2), encoding a transmembrane serine protease expressed not only in the inner hair cells but also in the spiral ganglion neurons and in the stria vascularis (Lee et al., 2013) were identified. Both affected siblings II:1 and II:2 are compound heterozygous for the two novel alleles, while parents I:1 and I:2 are healthy carriers (Fig. 3). The missense mutations c.1019C>G and c.1291C>T cause amino acid substitutions p.T340R and p.P431S, respectively, both located within TMPRSS3 protein serine protease domain, a highly conserved region across species (Fig. 3). Polyphen-2, SIFT and MutationTaster predict these mutations as damaging (Table 1). Family 1 is clinically characterized by a severe to profound ARNSHL mainly affecting the high-frequency audiometric thresholds (Fig. 4). Both

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