Confirmation of GRHL2 as the Gene for the DFNA28 Locus

Barbara Vona, Indrajit Nanda, Cordula Neuner, Tobias Müller, and Thomas Haaf*

1Institute of Human Genetics, Julius Maximilians University, Würzburg, Germany
2Department of Bioinformatics, Julius Maximilians University, Würzburg, Germany

Manuscript Received: 14 February 2013; Manuscript Accepted: 14 April 2013

More than 10 years ago, a c.1609_1610insC mutation in the grainyhead-like 2 (GRHL2) gene was identified in a large family with nonsyndromic sensorineural hearing loss, so far presenting the only evidence for GRHL2 being an autosomal-dominant deafness gene (DFNA28). Here, we report on a second large family, in which post-lingual hearing loss with a highly variable age of onset and progression segregated with a heterozygous non-classical splice site mutation in GRHL2. The c.1258-1G>A mutation disrupts the acceptor recognition sequence of intron 9, creating a new AG splice site, which is shifted by only one nucleotide in the 3’ direction. cDNA analysis confirmed a p. Gly420Glufs/C311 frameshift mutation in exon 10.

How to Cite this Article:
Vona B, Nanda I, Neuner C, Müller T, Haaf T. 2013. Confirmation of GRHL2 as the gene for the DFNA28 locus. Am J Med Genet Part A 161A:2060–2065.

Key words: autosomal dominant hearing impairment; DFNA28; GRHL2; haploinsufficiency; postlingual hearing impairment; progressive hearing loss

INTRODUCTION

Autosomal dominant nonsyndromic hearing impairment accounts for approximately 20% of hereditary hearing loss. To date, there are 54 autosomal dominant loci with 27 associated causative genes identified [Van Camp and Smith, 2012]. The DFNA28 (OMIM: 608641) locus is comprised of GRHL2 (OMIM: 608576) with the alias TFCP2L3 (transcription factor cellular promoter 2-like 3), which is a widely expressed transcription factor in human epithelial tissues [Werth et al., 2010]. GRHL2 spans approximately 177 kb on chromosome 8q22.3 (NCBI 37/hg19) and contains 16 exons, which translate into a 625 amino acid protein. It was first associated with the DFNA28 locus through mapping studies involving a five-generation North American family affected with mild to moderate post-lingual progressive bilateral sensorineural hearing loss. In this family, affected members had a heterozygous c.1609_1610insC mutation in exon 13 [Peters et al., 2002]. In addition, several single nucleotide polymorphisms (SNPs) in GRHL2 have been associated with marginal significance with age-related hearing impairment susceptibility [Van Laer et al., 2008]. Considering that a second disease-causing mutation has not been reported, one might begin to suspect that GRHL2 is not a bona fide deafness gene.

The expression and function of GRHL2 have previously been investigated in animal studies. Northern blot and in situ hybridization studies in the mouse demonstrated high Grhl2 expression in the cochlear duct at embryonic day 18.5 and postnatal day 5 [Peters et al., 2002; Wilanowski et al., 2002]. Grhl2−/− knockout mice were embryonic lethal, displaying split face and neural tube defects. Grhl2+/−/Grhl3+/− compound heterozygotes were viable and exhibited neural tube defects of varying severity. Evidently, coordinated expression of GRHL transcription factors in the non-neural ectoderm is important for neural tube closure [Rifat et al., 2010]. Unfortunately, hearing was not tested in heterozygous animals. Tol2 transposon-mediated insertional mutagenesis in zebrafish produced offspring with enlarged otocysts, reduced or absent otoliths, malformed semicircular canals, insensitivities to sound stimulation, and abnormal swimming position despite the normal appearance of hair cells in the inner ear. Upon wild type human GRHL2 mRNA injection, the inner ear defects in the zebrafish were rescued, whereas injection with mutant human GRHL2 was unable to rescue otic defects [Han et al., 2011]. This suggests a conserved structure and function of GRHL2 in vertebrate inner ear development.
MATERIALS AND METHODS

The study was approved by the Ethics Committee of the University of Würzburg.

Mutation Analysis

Genomic DNA was extracted from whole blood using a standard salt extraction method, and was submitted to Otogenetics Corporation (Norcross, GA) for exome capture (targeting 80 known deafness genes) and next generation sequencing (NGS) on a HiSeq2000 (Illumina, San Diego, CA). Paired-end reads of 90–100 bp were analyzed for quality, exome coverage, and exome-wide SNP/InDels using the platform provided by DNAnexus (Mountain View, CA), to which we applied our systematic analysis beginning with the removal of calls that did not meet certain quality and confidence thresholds. Intronic variants not predicted to affect splicing or regulation were also removed, since they are not likely to impact protein structure and function. As we expected the causative dominant mutation to be absent in the healthy population, it is unlikely to be reported in variant databases such as dbSNP and SwissVar. We also used SIFT [Ng and Henikoff, 2001], PolyPhen-2 [Adzhubei et al., 2010], and MutationTaster [Schwarz et al., 2010] to predict the impact of any identified amino acid substitution on the protein structure and function and to predict disease causing potential resulting from sequence alterations.

To validate the identified mutation, an amplicon containing the GRHL2 c.1258-1G>A mutation was PCR amplified from genomic DNA using standard PCR cycling conditions with forward primer 5’-GGATTTCACTGGTTTAGGG-3’ and reverse primer 5’-AGCGTAGACTTTCAAGTGAGC-3’ (Metabion, Martinsried, Germany). PCR products were sequenced with an ABI 3130xl 16-capillary sequencer (Life Technologies, Carlsbad, CA).

RNA Analysis

RNA samples were isolated from saliva using a standard protocol from the Oragene RNA collection kit (DNA Genotek, Ottawa, ON, Canada). RNA quality and quantity were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was produced using the SuperScript III First-Strand Synthesis SuperMix RT-PCR kit (Invitrogen, Karlsruhe, Germany). The GRHL2 region of interest was amplified using standard PCR cycling conditions from the synthesized cDNA with forward primer 5’-GGAAAA-TACTGGCACTCTCG-3’ and reverse primer 5’-ACCTTCTCGTC-ATCATCC-3’. A second round nested PCR continued with inner forward primer 5’-CCGTGAATTGCTTGAGCACA-3’ and reverse primer 5’-GGTTTGCAAAGTGAACATCAG-3’ in order to shorten the amplicon length for Sanger sequencing and enhance the product on the agarose gel.

RESULTS

Pedigree Analysis

The index patient (IV:4) developed type I diabetes at the age of 10 and bilateral progressive hearing loss at the age of 32. He does not show any syndromic features and maintains an academic position.

![Pedigree of the family. Black and purple filled symbols indicate family members with bilateral hearing impairment, light blue symbols individuals with an unclear hearing status. Approximate time of onset is denoted by black color, representing middle to late adulthood onset, and purple color, representing childhood onset. Individuals who were tested positively and negatively for the heterozygous GRHL2 mutation are indicated by a “+” or “−”, respectively. The index patient IV:4 is marked by an arrow. His 44-year-old sister IV:2 who also carries the mutation has normal hearing thresholds with exceptions at higher frequencies in the right ear. The inset shows the sequence chromatogram of parts of GRHL2 intron 9 and exon 10 of our index patient, containing the c.1258-1G>A substitution (highlighted in yellow).](image-url)
The family history allowed for the tracing back of hearing status over five generations (Fig. 1). Ten family members representing the last three generations were available for genetic analysis.

The hearing loss in the family members with detailed clinical examination is characterized as bilateral and progressive, usually beginning in the fifth decade of life (III:2, III:4, and IV:3) with the earliest documented age of diagnosis being 32 years of age in the index patient (IV:4) and latest diagnosis at age 65 (III:3). However notably, several more distantly related individuals without detailed clinical records (I:3, II:4, II:5, II:10, III:6, III:8, III:10, and III:11) had post-lingual childhood onset reported. Apart from childhood onset hearing loss, III:6 had a reduced IQ and severe epilepsy, which were thought to represent comorbidities contributing to his early death at approximately 40 years of age. Individuals III:8, III:10, and III:11 presented with varying spectrums of communication disorders in addition to childhood onset hearing loss and died prematurely at approximately 50 years of age. III:8 was described as having profound hearing impairment beginning from childhood and had infantile seborrheic dermatitis. III:10 was reported as having profound hearing loss that made it nearly impossible to communicate with him. III:11 was able to communicate orally with others, but overcompensated for his hearing loss by utilizing stilted speech.

Although great care was taken to record the hearing statuses of distantly related family members, there were three individuals having ambiguous hearing classifications. I:2 died at approximately 50 years of age at a time when his affection status was not clear. I:5 was reported as being hearing impaired; however, detailed information about onset and severity was unknown. Both these individuals lived in the 19th century, limiting clinical information to what members of the family collected. II:8 had normal hearing early in life but adult onset hearing loss cannot be excluded. III:12 was reported to hear normally, while his daughter IV:8 had mild hearing impairment at approximately 50 years of age. Thus, III:12 may be a non-penetrant mutation carrier. Unfortunately, III:14 and IV:8 were not available for genetic analysis and, thus, we could not test whether the same form of hearing loss segregates in the left and the right side of the pedigree.

Age of Onset and Progression of Hearing Loss

Figure 2 shows the bilateral pure-tone air conduction audiograms for family members III:2, III:3; III:4, IV:2, and IV:4. Hearing loss in all frequencies was observed for III:2, III:3, and III:4. Upward sloping profiles in these individuals indicate a greater affection in higher frequencies, particularly at 6 and 8 kHz, as compared to the lower and middle frequencies. IV:2 had only one recorded audiogram from age 44 and had normal hearing thresholds with exceptions at 6 and 8 kHz in the right ear. While IV:4 followed a predictable trend of hearing loss, he displayed an earlier and more severe onset, and was the only affected family member with type 1 diabetes.

Excluding individual IV:2, there was a positive correlation of hearing loss progression and advancing age exceeding what can be expected by normal aging (Fig. 3). We performed a linear regression analysis using the R statistical package [R Development Core Team, 2012] to estimate the progression of hearing loss. Figure 3A shows three of the five mutation carriers clustering linearly, two of which, namely III:2 and III:3, have closely matching
annual threshold deterioration (ATD) rates of 1.69 and 1.52 dB/year, respectively, when comparing hearing loss over age after averaging left and right ear thresholds and all frequencies. One outlier measurement was excluded for ATD calculation in III:2 at 54 years of age. Individual III:4 demonstrated greater hearing loss with his initial measurement and had a reduced ATD compared to his other family members with a value of 0.76 dB/year. The index patient IV:4 had an earlier and more severe hearing loss, with an ATD of 2.41 dB/year and when comparing the left and right thresholds averaged across all frequencies, he demonstrated greater hearing loss in his right ear compared to his left as seen in his last three measurements (Fig. 3B). When assessing lateralization of hearing loss using averaged frequencies, we were able to infer from individuals III:2, III:3, and III:4 that there was not a consistent lateral bias between left and right sided hearing loss (Fig. 3B). Apart from IV:2, affected individuals showed a mild (20–40 dB) to moderate (40–55 dB) sensorineural hearing loss in the fourth to seventh decade of life that progressed to moderately severe (55–70 dB) levels in higher frequencies by the seventh and eighth decade.

**Mutation Identification and Characterization**

The index patient was negative for mutations in the GJB2 (OMIM: 121011) gene. He was included in a microarray screen of 50 GJB2 mutation-negative non-syndromic hearing loss patients, which did not identify any potentially pathogenic copy number variations (data not shown). We then used targeted deafness gene enrichment sequencing (Otogenetics Corporation) to screen for mutations in 80 known deafness genes including 23 DFNA genes, 32 DFNB genes, and 2 DFN genes (with a number of genes being classified as being both dominant and recessive). Syndromic deafness genes were also included. The analysis strategy we employed filtered out apparently non-pathogenic variants, disclosing a single heterozygous c.1258-1G>A substitution in the GRHL2 gene as pathogenic. The average coverage of GRHL2 in the analyzed data set was 195x. It is worth emphasizing that mutations in the gene responsible for Wolfram syndrome (WFS1), which is characterized by juvenile diabetes mellitus, optic atrophy and progressive hearing loss, were excluded, as WFS1 is also covered in the NGS deafness panel. To date, we have analyzed 24 additional GJB2 mutation-negative hearing loss patients and eight normal hearing controls using targeted deafness gene sequencing and did not find any additional mutation in GRHL2 (data not shown).

Sanger sequencing of genomic DNA (accession: NG_011971.1) showed that the mutation was detected in five family members (III:2, III:3, III:4, IV:3, and IV:4) with middle to late adulthood onset of hearing loss and absent in the normal hearing father (III:1) of the index patient (Fig. 1). Individual IV:2 who was also heterozygous for the GRHL2 mutation did not report hearing loss at the age of 44. In the youngest generation, we identified one heterozygous individual (V:1) and two individuals (V:2 and V:3) without the mutation. Hearing was normal in all three of these individuals, which was expected, considering their young age.

We initially predicted that the c.1258-1G>A substitution in the GRHL2 intron 9 would result in the skipping of exon 10. To test this, we extracted RNA from saliva samples from the index patient and a normal hearing control, synthesized cDNA (accession: NM_024915.3), and amplified a region spanning exons 9 and 11. Comparing the product size of the patient and control through gel electrophoresis, it was demonstrated that the exon 10 was not skipped (Fig. 4A). Instead, Sanger sequencing of the cDNA product showed that a new 3’ AG splice site was shifted by only one nucleotide in the 3’ direction, causing a heterozygous deletion of the first guanine in exon 10 (Fig. 4B). This mutation thus predicts a p.Gly420Glufs*111 in exon 13 (Fig. 4C).
DISCUSSION

We report on a second DFNA28-causing mutation and the first splice site mutation in GRHL2 in a family affected with non-syndromic hearing loss. Previously, only one mutation in GRHL2 has been associated with hearing loss [Peters et al., 2002]. The mutation described here confirms that mutations in GRHL2 cause postlingual progressive hearing loss. In this light, it may also be worth following up the marginally significant association of presbycusis with GRHL2 variants [Van Laer et al., 2008], using larger cohorts.

This newly identified GRHL2 mutation constitutes a type IV nonclassical (intronic) splicing mutation, which could have been misinterpreted as a classic (type I) splice defect if cDNA was not analyzed [Eng et al., 2004]. In the index patient, the heterozygous c.1258-1G>A mutation introduces a new 3’ AG splice site that causes a deletion in the first nucleotide of exon 10. The splice site is composed of the mutant A and the wild type G in the first position of exon 10. The red nucleotide represents the G>A mutation in intron 9. Partial intron 9 and exon 10 sequence is boxed and depicted in lowercase and capital letters, respectively. The mutated position is highlighted in red and the deleted nucleotide is underlined.

Grhl2 participates in the differentiation and maintenance of epithelial cells throughout life [Werth et al., 2010]. Impaired
epithelial cell integrity is the most reasonable pathological explanation as to its involvement in late-onset hearing impairment [Peters et al., 2002; Van Laer et al., 2008]. Considering a number of factors that are useful for predicting haploinsufficiency such as temporal expression, proximity to other haploinsufficiency genes, interaction partners, and genetic implication in disease [Huang et al., 2010], GRHL2 is predicted to have a high probability of exhibiting haploinsufficiency. It is plausible to assume that the hearing loss in the present family and the previously reported family [Peters et al., 2002] is due to GRHL2 haploinsufficiency.

The results show that the heterozygotes for the c.1258-1G>A mutation in GRHL2 have progressive, bilateral hearing loss with a typical onset in middle to late adulthood. The variability in the onset of hearing loss and audiometric profiles in heterozygotes argue for the interplay of other genetic or environmental factors in determining the events leading to hearing loss. Comorbidities independent of hearing loss such as epilepsy, reduced IQ, and type 1 diabetes may influence the onset and severity of hearing loss and explain this variation. Alternatively, given the enormous genetic heterogeneity of hearing loss and the high rate of marriage among hearing-impaired individuals, it is possible that the family members with a documented childhood onset in the right branch of the pedigree, who were not available for genetic diagnostics and detailed clinical examination, suffer from a distinct form of dominant deafness.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to the family who has actively participated in this study. We also thank Dr. Andrea Gehrig for helping with the mutation analyses, Dr. Jörg Schröder for helping with the collection of audiograms, and Claus Steinlein for technical assistance. The authors greatly appreciate the journal editor’s and reviewers’ support and helpful comments to the original manuscript. T.H. was supported by a grant (HA 1374/7-2) from the German Research Foundation.

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