A Novel Splice-Site Mutation in the GJB2 Gene Causing Mild Postlingual Hearing Impairment

Marta Gandía1,2, Francisco J. del Castillo1,2, Francisco J. Rodríguez-Álvarez1,2, Gema Garrido1,2, Manuela Villamar1,2, Manuela Calderón3, Miguel A. Moreno-Pelayo1,2, Felipe Moreno1,2, Ignacio del Castillo1,2*

1 Unidad de Genética Molecular, Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain, 2 Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain, 3 Servicio de Otorrinolaringología, Hospital Universitario Ramón y Cajal, Madrid, Spain

Abstract

The DFNB1 subtype of autosomal recessive, nonsyndromic hearing impairment, caused by mutations affecting the GJB2 (connection-26) gene, is highly prevalent in most populations worldwide. DFNB1 hearing impairment is mostly severe or profound and usually appears before the acquisition of speech (prelingual onset), though a small number of hypomorphic missense mutations result in mild or moderate deafness of postlingual onset. We identified a novel GJB2 splice-site mutation, c. -22-2A>C, in three siblings with mild postlingual hearing impairment that were compound heterozygous for c. -22-2A>C and c.35delG. Reverse transcriptase-PCR experiments performed on total RNA extracted from saliva samples from one of these siblings confirmed that c. -22-2A>C abolished the acceptor splice site of the single GJB2 intron, resulting in the absence of normally processed transcripts from this allele. However, we did isolate transcripts from the c. -22-2A>C allele that keep an intact GJB2 coding region and that were generated by use of an alternative acceptor splice site previously unknown. The residual expression of wild-type connection-26 encoded by these transcripts probably underlies the mild severity and late onset of the hearing impairment of these subjects.

Introduction

Hereditary non-syndromic hearing impairment (NSHI) is a heterogeneous genetic condition. To date, mutations in 73 different genes have been shown to cause NSHI. At least as many genes remain to be identified, as indicated by the number of known NSHI loci for which the underlying causative mutations have not been found yet (http://hereditaryhearingloss.org/) [1]. This high genetic heterogeneity mirrors the structural and functional complexity of the hearing process and it is a major hurdle for the successful genetic diagnosis of subjects with NSHI.

The DFNB1 subtype of autosomal recessive NSHI is remarkable for being highly prevalent in most of the populations tested so far (reviewed in 2). The underlying locus, DFNB1, lies on 13q12 and encompasses the GJB2 gene (MIM # 121011), which encodes the gap-junction protein connection-26 (Cx26). GJB2 consists of a 160-bp non-coding exon, a single 3,179-bp intron and a 2,134-bp exon that contains 22 bp of the 5’-untranslated region (UTR), the complete 678-bp coding sequence and the 3’-UTR (UniGene Hs.524894) [3].

Mutations at the DFNB1 locus can be classified in two groups: (i) those that affect the coding sequence of GJB2, and (ii) those that lie outside the coding sequence of GJB2 and affect the expression and/or regulation of this gene [2]. Since screening of GJB2 is considered the gold standard of genetic diagnosis of hereditary HI, it is not surprising that more than 100 pathogenic mutations in the GJB2 coding sequence have been identified so far (http://davinci.crg.es/deafness/) [4]. A few GJB2 mutations predominate in particular populations due to demonstrated founder effects [5–11].

By contrast, only six pathogenic mutations are known outside the GJB2 coding sequence. Two of them are point mutations: c. -23+1G > A (originally named IVS1+1G>A), the only mutation known to affect the donor splice site of the single
intron [12], and g. -77C>T (originally named -3438C>T), which abolishes the activity of the basal promoter of the gene [13]. The remaining four mutations are large deletions. One of them is a deletion of about 920 kb that encompasses the complete GJB2 gene [14], whereas the three other deletions (del(GJB6-D13S1830) [15], del(GJB6-D13S1854) [16] and del(131-kb) [17], respectively) are thought to eliminate a hypothesized cis-acting regulatory element located far upstream of GJB2. While the 920-kb and del(131-kb) deletions and the g. -77C>T promoter mutation seem to be private mutations, the del(GJB6-D13S1830) and del(GJB6-D13S1854) deletions and the c. -23+1G > A splice-site mutation are frequent in specific populations [16,18–22]. All of these mutations have been isolated in compound heterozygosity with GJB2-coding sequence mutations.

DFNB1 hearing impairment is clinically heterogeneous because of intrafamilial and interfamilial phenotypic variability, even in association with a same genotype [23]. The most common form is prelingual (onset before the acquisition of speech), non-progressive and severe or profound, affecting all frequencies. However, postlingual, progressive and moderate or mild hearing losses have also been reported, often associated with a few specific mutations [23]. Identification of these genotype-phenotype correlations is important to improve the accuracy of genetic counselling.

In this work, we studied a Spanish pedigree with three siblings affected by a mild, postlingual NSHI. We identified a novel GJB2 mutation, which is the first one shown to alter the acceptor splice site of the single intron of the GJB2 gene. Investigation of its effects on GJB2 expression provided a likely explanation of the molecular mechanism underlying this mild DFNB1 phenotype.

Materials and Methods

Ethics statement

This study was approved by the Ethical Committee for Clinical Research of Hospital Universitario Ramón y Cajal. The study complied with the Spanish laws for biomedical research currently in force and adhered to the tenets of the Declaration of Helsinki.

Subjects and clinical tests

Written informed consent was obtained from all the subjects included in the study. Syndromic features or putative environmental causes of HI were excluded in all affected subjects. HI was evaluated by pure-tone audiometry, testing for air conduction (frequencies 125–8,000 Hz) and for bone conduction (frequencies 250–4,000 Hz). The degree of HI was determined by calculating the binaural mean of the hearing thresholds for air conduction at frequencies 0.5, 1, and 2 kHz, and it was classified as mild (average thresholds in the range of 21–40 dB), moderate (41–70 dB), severe (71–90 dB), or profound (>90 dB). The HI of subject III:2 was also evaluated by auditory brainstem response (ABR) recording.

DNA purification and assay procedures

DNA was extracted from peripheral blood samples by standard procedures. Genetic tests for mutations in the GJB2 gene and for the large deletions affecting the GJB6 gene at the DFNB1 locus were carried out as published [11,16]. Mutation nomenclature is based on cDNA sequence (GenBank accession number NM_004004.5) and follows current Human Genome Variation Society rules as implemented by the MUTALYZER 2.08 program (http://mutalyzer.nl).

The restriction fragment length polymorphism (RFLP) assay for the c. -22-2A>C mutation was carried out by digesting a 907-bp PCR product containing GJB2 exon 2 and the intron 1/exon 2 boundary (obtained with primers 5’-ACCTTTGT AGGGTTGTGT-3’ and 5’-TGATCACGGGTGCTCATC-3’), with restriction endonuclease EcoRI, as recommended (Fermentas). The mutation creates a unique EcoRI site. Digestion products were as follows: wild-type allele: 907 bp; c. -22-2A>C allele: 770 bp + 137 bp.

Analysis of splice sites in the GJB2 intronic sequence was performed by NNSPLICE software, version 0.9 (http://www.fruitfly.org/seq_tools/splice.html) [24].

RNA purification and assay procedures

Saliva samples were collected in Oragene RNA Self-Collection Vials (DNA Genotek) according to the instructions of the manufacturer and stored at -20°C. Total RNA was extracted from 1 mL saliva samples following the Oragene RNA Purification protocol (DNA Genotek) with an RNeasy Micro kit (Qiagen). The protocol includes a 15-min incubation step with RNase-free DNase I (Qiagen) to remove contaminating DNA. cDNA was synthesized with 500 ng total RNA as template and random hexamer primers by using Superscript II reverse transcriptase (Life Technologies) as recommended.

To identify GJB2 splicing products, we amplified cDNA by PCR by using FastStart DNA polymerase with GC-Rich Solution (Roche) and the following procedure: template cDNA was denatured for 5 min at 95°C, followed by 40 cycles of denaturation at 94°C (30 sec), annealing at 60°C (30 sec) and extension at 72°C (30 sec), and a final extension step at 72°C for 7 min. GJB2-specific primers were designed in exon 1 (forward primer: 5’-CGCGCTCCTCTCCTCC-3’) and exon 2 (reverse primer: 5’-CTCCTTGAGCCACAAACGGAGAT-3’) to rule out amplification of any contaminating genomic DNA. The reverse primer was used either unlabelled or labelled with 6-FAM at the 5’ end. Labelled PCR products were treated with T4 DNA polymerase (Roche) to blunt any 3’ protruding termini and were subsequently resolved by capillary electrophoresis in an ABI Prism 3130 Genetic Analyzer (Life Technologies). Unlabelled PCR products were separated in a 1.5% SeaKem GTG agarose gel; bands were excised and DNA was recovered by using NucleoSpin Extract II (Macherey-Nagel). Gel-extracted PCR products were either directly sequenced or cloned in the T-vector pCR2.1-TOPO with the TOPO TA Cloning kit (Life Technologies) and subsequently sequenced.

Quantification of GJB2 splicing products was performed by real-time quantitative PCR (RT-qPCR) in a 7300 Real Time PCR System (Life Technologies). Specific primer pairs were
A novel splice-site mutation in GJB2

We ascertained a family with nonsyndromic sensorineural HI that apparently segregated with an autosomal dominant inheritance pattern (family S1599; Figure 1A). Clinical presentation of the HI was heterogeneous: subject III:2, the index case, had severe prelingual deafness, whereas his father (II:4) and two paternal aunts (II:1 and II:6) had mild HI of postlingual pattern and presented intra-familial phenotypic variability. Interestingly, the phenotype of mild postlingual HI was observed three labelled PCR products of 278, 313 and 317 bp (Figure 3B). The absence of a 279-bp product confirmed that the two splice sites were indeed used, albeit with different efficiencies, as indicated by the much larger peak corresponding to use of the known splice site.

For the assay, the reverse primer was labelled with a fluorophore, and blunted PCR products were resolved in a capillary fragment analyzer. We expected a 279-bp product if the known splice site was used and a 317-bp product in case that the alternative splice site was used. We verified that RNA obtained from saliva samples was a source of GJB2 transcripts. We amplified these transcripts by reverse-transcriptase PCR techniques to assay for the expression of GJB2. Forward and reverse PCR primers were located in exons 1 and 2, respectively. The amplification product included the site of the c.35delG mutation, in order to distinguish the c.-22-2A>C allele from the c.35delG one (which would be 1-bp shorter). For the assay, the reverse primer was labelled with a fluorophore, and blunted PCR products were resolved in a capillary fragment analyzer. We expected a 279-bp product if the known splice site was used and a 317-bp product in case that the alternative splice site was used. We verified that RNA obtained from saliva samples was a source of GJB2 transcripts. We amplified these transcripts by reverse-transcriptase PCR techniques to assay for the expression of GJB2. Forward and reverse PCR primers were located in exons 1 and 2, respectively. The amplification product included the site of the c.35delG mutation, in order to distinguish the c.-22-2A>C allele from the c.35delG one (which would be 1-bp shorter).

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bands (278, 313, and 317 bp). Bands were excised from the gel and purified (the 313 and 317 bp bands were purified together as it was not possible to excise them separately because of their proximity in the gel). Purified PCR products were directly sequenced; in parallel, the products were cloned in a T-vector and individual clones were sequenced. Sequencing showed that the 278/279 bp and 317 bp fragments did correspond to the transcripts generated by use of the known site and the alternative site 1, respectively (Figure 4).

Figure 1. Hearing impairment in family S1599. A Pedigree of family S1599, indicating the GJB2 genotypes of subjects who were analyzed. B Audiograms (air conduction) of affected individuals II:1 and II:6 at ages 50 and 31 years, respectively (circles, right ear; crosses, left ear). The binaural means of the hearing thresholds for air conduction at frequencies 0.5, 1, and 2 kHz for these subjects are 33.3 and 25.8 dB, respectively, corresponding to mild HI (21-40 dB). doi: 10.1371/journal.pone.0073566.g001
the alternative site 1 added 38 intronic nucleotides to the transcript, while use of the alternative site 2 just added 34 intronic nucleotides (Figure 2). The alternative site 2 is less used than site 1, as indicated by the assay performed in the capillary fragment analyzer (Figure 3B).

We quantified the relative amounts of GJB2 cDNAs from subject II:4 by means of real-time qPCR. Data were normalized by comparing with data obtained from a control subject and referred to the levels of a ubiquitously expressed control gene (GAPDH). For these assays, primers were designed for the specific amplification of cDNAs obtained by splicing from the known site and the alternative site 1. The ratios for each transcript between II:4 and the wild-type control were 0.18 (known site) and 1.68 (alternative site 1) (Figure 5). Since the c. -22-2A>C mutation abolishes the known acceptor site, we expected that the amount of transcript from this site in subject II:4 (generated from the c.35delG allele) was about 50% that of the control. However, it is just 18%, which suggests that transcripts with c.35delG may be either less stable or less efficiently spliced than wild-type transcripts. In addition, our

Figure 2. Sequence of the exon-intron junctions of the single GJB2 intron. Exonic sequence is shown in capitals, whereas intronic sequence is shown in lowercase italics. The three acceptor splice sites identified in this work appear boxed in bold: KS, known acceptor site; AS1, alternative site 1; AS2, alternative site 2. The ATG start codon appears in bold. Nucleotides affected by the c. -22-2A>C and c.35delG mutations are indicated by arrows. Locations of the primers used for identifying GJB2 splicing products are underlined.
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Figure 3. Analysis of GJB2 splicing products. Splicing products from transcription of GJB2 were amplified in a fluorescent RT-PCR assay performed with total RNA extracted from saliva samples and were separated by capillary electrophoresis. A GJB2 splicing products from a normal-hearing control (genotype wt/wt). B GJB2 splicing products from c. -22-2A>C/c.35delG compound heterozygous subject II:4.
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results indicate that abolition of the known site results in an increased use of alternative site 1.

Discussion

We have identified a novel GJB2 splice-site mutation (c. -22-2A>C) which belongs to the small group of DFNB1-causing mutations that lie outside the coding region of the GJB2 gene. Only another pathogenic splice-site mutation, c. -23+1G > A [12], had been identified to date in this simple gene containing just two exons. The c. -23+1G > A and c. -22-2A>C mutations abolish the donor and acceptor splice sites of GJB2 intron 1, respectively. Unlike many splice-site mutations, they have no direct effect on protein coding because the coding sequence of GJB2 is completely contained within exon 2. Rather, their pathogenic effect is due to impaired expression of the mutant alleles. As regards c. -23+1G>A, GJB2 transcripts generated from this allele were not detected in a lymphoblastoid cell line derived from a c. -23+1G>A/c.35delG compound heterozygote [28], suggesting that either the c. -23+1G > A allele was not transcribed or the transcript was unstable. This result agreed with the profound HI of this subject. Indeed, the c. -23+1G > A mutation is nearly always associated with severe or profound HI of prelingual onset when in homozygosity or in compound heterozygosity with truncating mutations such as c.35delG, c.167delT, c.235delC and c.290dupA [22,29–32]; it was reported in association with mild or moderate HI of postlingual onset. The c. -22-2A>C mutation seems to belong to this group of hypomorphic alleles given the hearing phenotype observed in the three affected siblings of family S1599 (mild HI of late postlingual onset). This correlation ought to be regarded as provisional, as it has been deduced from just three siblings. Yet, the lack of phenotypic variability among the affected siblings and the observed effects of c. -22-2A>C on GJB2 expression support this conclusion.

The c. -23+1G > A mutation is the most frequent DFNB1 pathogenic allele in Mongolia [22] and a relatively frequent allele in the Kurdish, Turkish, Czech, Polish and Chinese populations [19–21,32,33]. On the contrary, c. -22-2A>C seems to be a relatively rare allele. Our screening procedure does not miss this mutation because we sequence both GJB2 exons and exon-intron junctions in all cases in which DFNB1 HI is suspected. However, c. -22-2A>C is first reported here, and it has only been found once in our large cohort of HI cases from the Spanish population, which is consistent with its low allelic frequency (0.00065) in Caucasian populations, as reported by the 1000 Genomes and NHLBI Exome Sequencing projects. The fact that it has not been found in any other cohorts of subjects with HI studied so far might be just the consequence of an enrolment bias towards subjects with severe or profound HI, which would under-represent hypomorphic alleles associated with late-onset mild-to-moderate HI. Although we did not find the mutation in a few cases with presbycusis, a
Figure 5. Relative expression levels of GJB2 transcripts generated by splicing at different acceptor sites. Each panel shows results for a normal-hearing control (genotype wt/wt) and for c.-22-2A>C/c.35delG compound heterozygous subject II:4. A Relative expression of GJB2 transcript generated by splicing from the known site (two-tailed $p < 0.0001$, N=5). B Relative expression of GJB2 transcript generated by splicing from alternative site 1 (two-tailed $p=0.0094$, N=3).

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hypothesised involvement of c.-22-2A>C in this pathology deserves further exploration.

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Author Contributions

Conceived and designed the experiments: MG FJdC FJRA IdC. Performed the experiments: MG FJRA GM MV MC. Analyzed the data: MG FJdC FJRA IdC. Contributed reagents/materials/analysis tools: MAMP FM IdC. Wrote the manuscript: MG FJdC MAMP IdC.
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