Allele-Specific Impairment of GJB2 Expression by GJB6 Deletion del(GJB6-D13S1854)

Juan Rodriguez-Paris1, Marta L. Tamayo2,3, Nancy Gelvez2, Iris Schrijver1,4*

1 Department of Pathology, Stanford University School of Medicine, Stanford, California, United States of America, 2 Instituto de Genética Humana, Universidad Javeriana, Bogotá, Colombia, 3 Fundación Oftalmológica Nacional, Bogotá, Colombia, 4 Department of Pediatrics, Stanford University School of Medicine, Stanford, California, United States of America

Abstract

Mutations in the GJB2 gene, which encodes connexin 26, are a frequent cause of congenital non-syndromic sensorineural hearing loss. Two large deletions, del(GJB6-D13S1830) and del(GJB6-D13S1854), which truncate GJB6 (connexin 30), cause hearing loss in individuals homozygous, or compound heterozygous for these deletions or one such deletion and a mutation in GJB2. Recently, we have demonstrated that the del(GJB6-D13S1830) deletion contributes to hearing loss due to an allele-specific lack of GJB2 mRNA expression and not as a result of digenic inheritance, as was postulated earlier. In the current study we investigated the smaller del(GJB6-D13S1854) deletion, which disrupts the expression of GJB2 at the transcriptional level in a manner similar to the more common del(GJB6-D13S1830) deletion. Interestingly, in the presence of this deletion, GJB2 expression remains minimally but reproducibly present. The relative allele-specific expression of GJB2 was assessed by reverse-transcriptase PCR and restriction digestions in three probands who were compound heterozygous for a GJB2 mutation and del(GJB6-D13S1854). Each individual carried a different sequence variant in GJB2. All three individuals expressed the mutated GJB2 allele in trans with del(GJB6-D13S1854), but expression of the GJB2 allele in cis with the deletion was almost absent. Our study clearly corroborates the hypothesis that the del(GJB6-D13S1854), similar to the larger and more common del(GJB6-D13S1830), removes (a) putative cis-regulatory element(s) upstream of GJB6 and narrows down the region of location.

Introduction

The DFNB1 locus at chromosome 13q11-q12 includes the GJB2 and GJB6 genes, which respectively encode connexin 26 (Cx26) and connexin 30 (Cx30). These connexin proteins are co-expressed and co-localized in the cochlea, where they create heteromeric gap junctions [1] and make important contributions to cochlear homeostasis [2]. GJB2 mutations are the most common genetic etiology of prelingual non-syndromic sensorineural hearing loss [3,4]. Even though many recessive GJB2 mutations have been described (http://davinci.crg.es/deafness/), so far only four recessive mutations affecting GJB6 or the region upstream have been reported, all of which are deletions [5-12] (Fig 1). The most common are del(GJB6-D13S1830) and del(GJB6-D13S1854), which truncate the GJB6 gene [5-9]. The other two are private mutations, one of which (>930 kb) deletes both GJB2 and GJB6 [11] and the other (del(chr13:19,837,343–19,968,698) does not affect either gene and is located upstream of GJB6 [10,12] (Fig 1).

We have recently reported that the larger of the two most common deletions del(GJB6-D13S1830), disrupts human GJB2 expression at the transcriptional level in an allele-specific manner [13], presumably by removing one or more cis-regulatory elements located within the deleted region, which are as yet obscure. Given the large proportion (up to ~13% in the USA [14]) of individuals left with only one recessive GJB2 mutation after genetic testing instead of the two expected, it seems plausible that some of the unidentified mutations could be cis-regulatory in nature and would be located outside the transcribed region.

The goal of this study was to determine whether the smaller and less common del(GJB6-D13S1854) deletion, similar to del(GJB6-D13S1830), disrupts human GJB2 expression at the transcriptional level, which would lend further support to the putative presence of cis-regulatory element(s) upstream of GJB6. It has been assumed that del(GJB6-D13S1834) and del(GJB6-D13S1830) would affect GJB2 expression similarly, however until now this has never been demonstrated. Our study also sought to narrow down the region were the supposed cis-regulatory element(s) may be located, based on the fact that del(GJB6-D13S1834) is ~77 kb smaller than del(GJB6-D13S1830) and that it is included within that deletion. In order to investigate the relative abundance of GJB2 transcripts from each allele, allele-specific analyses based on reverse-transcriptase PCR (RT-PCR) were conducted with RNA extracted from buccal epithelial cells, which are known to express both Cx26 and Cx30 [10,13]. In all three probands, each of whom carried a different sequence variant in GJB2, the expression of GJB2 from the chromosome bearing the del(GJB6-D13S1854) deletion was virtually absent (Table 1).
Materials and Methods

Study subjects
Three controls with neither del(GJB6-D13S1854) nor del(GJB6-D13S1830) and five probands were included in this study. Three of the affected individuals were of Colombian origin (CE1, CE2 and CE3) as were two of the controls (CTR2 and CTR3). One proband (CE1) was homozygous for del(GJB6-D13S1830), one carried del(GJB6-D13S1830) and a GJB2 mutation in trans (CE2), and three were compound heterozygous for del(GJB6-D13S1854) and a GJB2 mutation in trans (CE3, CE4 and CE5). This study was approved by the Stanford University institutional review board, and written informed consents were obtained from all participants (Table 1). The participating probands had hearing loss ranging from moderate to profound. Control CTR1 had no identified sequence changes; the other two controls were related to probands and were heterozygous carriers of the GJB2 Ser199Phe (596C>T) mutation. Control CTR2 was the father of proband CE2 and control CTR3 was the mother of proband CE3. None of the controls were affected. Direct sequencing and a restriction fragment length polymorphism (PCR-RFLP) assay identified or confirmed all sequence changes.

RT-PCR
Total RNA was isolated from buccal epithelium cells as previously described [13]. Briefly, cells were collected on Cytosoft® Plus brushes (CooperSurgical, Trumbull, CT) and immediately submerged in RNAlater® (Ambion, Austin, TX). Cells were spun down and total RNA was extracted following the RNAeasy mini kit protocol (Qiagen, Valencia, CA). cDNA synthesis was performed according to the SuperScript III (Invitrogen, Carlsbad, CA) reverse transcription method and a negative control to rule out gDNA contamination was included for each sample. The negative controls followed all procedure steps with the matching samples and remained negative (data not shown). We used cDNA-specific primers to avoid amplification from gDNA. First, a forward primer in GJB2 exon 1 was paired with a reverse primer in exon 2 to generate a 728 bp PCR product from the cDNA template only [13,15]. These primers amplify a region enclosing the whole GJB2 coding sequence (CDS) (Table 2, Fig 2A). β-actin primers specific for cDNA [16] were used as an RNA expression control for the sample homozygous for the del(GJB6-D13S1830) deletion (CE1, Table 1). Second, for evaluation in the presence of the 35delG mutation we used primers designed by Wilch et al and Wilcox et al. [10,17], which were located in exons 1 and 2, as well. These primers generated a smaller (139 bp) PCR product, also however specific only to cDNA (Table 2, Fig 3A). Four μl cDNA per 20 μl reaction and 40 amplification cycles were used for all the PCR amplifications described above.

Allele-specific assays
Relative transcript abundance from each GJB2 allele was estimated by RT-PCR and RFLP assays. In the instance of GJB2 change Gln80Pro (239A>C), the 728 bp amplicon from the GJB2

![Figure 1. Map of the region on chromosome 13q11-12 disrupted by deletions.](image)

The ~365 kb DNA section encompasses three genes (dark boxes), the breakpoints of the deletions (arrowheads) and their range (dashed lines). All elements are drawn approximately to scale. GJB6 and CRYL1 are impacted by three of the four deletions; however GJB2 is directly affected only by the largest deletion. del(GJB6-D13S1854) and del(GJB6-D1351830) are investigated in this study. The transcriptional start sites are shown by bent arrows. doi:10.1371/journal.pone.0021665.g001

Table 1. Genotypes and phenotypes of study subjects.

| Proband | GJB2 variant(s) | GJB6 variant(s) | Phenotype                |
|---------|----------------|----------------|-------------------------|
| CE1     | WT/WT          | Δ(GJB6-D13S1830) | Profound hearing loss   |
| CE2     | S199F/WT       | Δ(GJB6-D13S1830)/WT | Profound hearing loss   |
| CE3     | S199F/WT       | Δ(GJB6-D13S1854)/WT | Profound hearing loss   |
| CE4     | Q80P/WT        | Δ(GJB6-D13S1854)/WT | Severe hearing loss     |
| CE5     | 35delG/WT      | Δ(GJB6-D13S1854)/WT | Moderate to Severe hearing loss |
| Control | GJB2 variant(s) | GJB6 variant(s) | Phenotype                |
|---------|----------------|----------------|-------------------------|
| CTR1    | WT/WT          | WT/WT          | None                    |
| CTR2    | S199F/WT       | WT/WT          | None                    |
| CTR3    | S199F/WT       | WT/WT          | None                    |

doi:10.1371/journal.pone.0021665.t001
### Results

We determined the allele-specific expression of *GJB2* in four study subjects who were compound heterozygous for either del(*GJB6*-D13S1854) or del(*GJB6*-D13S1830) and a mutation in *GJB2*, in *trans* (Table 1). At least one parent of each proband was genotyped for both *GJB2* and *GJB6* to confirm that the mutations were indeed present on opposite alleles. We also analyzed the allele-specific expression of *GJB2* in a fifth individual (CE1) who was homozygous for the del(*GJB6*-D13S1830) deletion. Based on our previous results [13], this individual with profound hearing loss was expected to not express *GJB2* at all. The complete failure to amplify the 728 bp segment from the *GJB2* cDNA indeed indicates the complete absence of *GJB2* expression from both del(*GJB6*-D13S1830) alleles (Fig 4). *GJB2* expression from an unaffected individual with no identified mutations in either *GJB6* or *GJB2* (CTR1) was used as a control and the 728 bp segment was amplified as expected (Fig 4). cDNA-specific ß-actin expression [16] was used for RNA expression control. Both proband and control amplified the expected 626 bp segment, confirming normal ß-actin expression (Fig 4).

Study participant CE2 inherited the del(*GJB6*-D13S1830) deletion from his mother and the Ser199Phe mutation in *GJB2* from his father (Fig 2B). In order to make possible the distinction between the affected and wild-type *GJB2* alleles, we performed allele-specific restriction digestions. The complete and reproducible absence of the 100 and 101 bp bands associated with the wild-type allele for Ser199Phe (CE2, Fig 2D) demonstrated a lack of allele-specific restriction digestions. The complete and reproducible absence of the 100 and 101 bp bands associated with the wild-type allele for Ser199Phe (CE2, Fig 2D) demonstrated a lack of allele-specific restriction digestions. The complete and reproducible absence of the 100 and 101 bp bands associated with the wild-type allele for Ser199Phe (CE2, Fig 2D) demonstrated a lack of allele-specific restriction digestions. The complete and reproducible absence of the 100 and 101 bp bands associated with the wild-type allele for Ser199Phe (CE2, Fig 2D) demonstrated a lack of allele-specific expression from both *GJB6* allele (CTR1) and from the father of the proband who is a carrier of Ser199Phe (CTR2) were used as controls and were amplified as well as digested simultaneously with the sample from the proband (Fig 2D). Both controls expressed the expected 201 bp fragment associated with the wild-type allele.

### Table 2. PCR-RFLP used to distinguish *GJB2* variants Gln80Pro (239A>C), Ser199Phe (596C>T) and 35delG.

| Mutation | Primer and sequence (5’–3’ ) | Product size (bp) | Restriction enzyme | Fragment sizes (bp) |
|----------|-------------------------------|-------------------|-------------------|-------------------|
| Q80P     | aF: TCTTCTGGGCGGAGAGCAA       | 728               | PvuII-HF          | Wt: 451+277       |
|          | bF: GGGCAATAGTTAAACTGACC      |                   |                   | Mt: 728           |
| S199F    | aF: TCTTCTGGGCGGAGAGCAA       | 728               | AlwNI             | Wt: 527+101+100   |
|          | bF: GGGCAATAGTTAAACTGACC      |                   |                   | Mt: 527+201       |
| 35delG   | aF: CGCGAGAGCCCCAAAAGCGAGA    | 139               | BstN1             | Wt: 110+29        |
|          | bF: GCTGGGAAAGTTGGTTGTACACACACGGCA |                 |                   | Mt: 139           |

*Rodriguez-Paris et al. [13], Mueller et al. [15], Wilcox et al. [17]. Wt = wild-type; Mt = Mutant. doi:10.1371/journal.pone.0021665.t002

CDs was digested with PvuII-HF to avoid star activity (the digestion of sequences which are similar but not identical to the restriction enzyme’s unique recognition sequence). The variant eliminates a single PvuII-HF restriction site, which yields an undigested product (728 bp) instead of two fragments (451 and 277 bp) when this variant is absent (Table 2 and Fig 2A). Variant Ser199Phe (596C>T) eliminates an AlwNI restriction enzyme site, which limits digestion of the Ser199Phe allele product to two fragments (527 bp and 201 bp), instead of three (527, 101 and 100 bp) for the wild-type allele (Table 2 and Fig 2A).

For the 35delG mutation, a mismatched reverse primer [10] eliminates a BstN1 restriction site from the 139 bp RT-PCR product, and this allows differentiation between the mutant and wild-type alleles. The digested wild-type allele is associated with fragment sizes of 110 bp and 29 bp, whereas the amplicon associated with the mutant allele remains undigested (Table 2 and Fig 3A).

### Restriction enzyme digestion

Digestion was performed as previously described [13]. In brief, 10 μL of PCR product was digested with 10 units of the applicable restriction enzyme for two hours, according to the manufacturer’s protocol (New England Biolabs, Beverly, MA). Conditions conducive to star activity were avoided and high fidelity restriction enzymes were chosen when available. Products were electrophoresed on 3% agarose gels or on 3.5% NuSieve 3:1 agarose gels (Lonza, Rockland, ME).

### Sequence analysis of the basal promoter, exon 1 and the *GJB2* coding region

The basal promoter and exon 1 of the *GJB2* gene of three selected samples (CE4, CE5 and CTR1) were investigated for the presence of mutations according to Matos et al. [18]. In brief, a 1009 bp *GJB2* segment was amplified which included part of the 5’ UTR, exon 1 and the donor splice site of intron 1. A 539 bp segment from this amplicon was sequenced using a hemi-nested forward primer and the same reverse primer. This sequenced segment included the 128 bp proximal promoter region, exon 1 and its donor splice site. The *GJB2* CDS, the flanking sequence of intron 1 (including the acceptor splice site) and a fraction of the 3’UTR were also sequenced for all participants.
Figure 2. Allele-specific expression analysis for GJB2, based on Ser199Phe (596C>T) and Gln80Pro (239A>C). (A) Schematic design of AlwNI and Pvull-HF digestion analysis. A 728 bp GJB2 cDNA amplicon was digested with AlwNI to distinguish Ser199Phe (5199F) from the wild-type allele, or with Pvull-HF to distinguish Gln80Pro (Q80P). Bars indicate GJB2 exons. The translated region within exon 2 is shaded. The relative positions of the GJB2 sequence changes (dotted arrows) and restriction sites (arrows) are indicated. Digestion fragment sizes are listed for both sequence changes and the wild-type. (*) and (‘) indicate fragments unique to the wild-type alleles in cis with del(GJB6-D13S1830) or del(GJB6-D13S1854), respectively. (B) Pedigree of proband CE2, who carries del(GJB6-D13S1830) in trans with Ser199Phe in GJB2. (C) Pedigree of proband CE3, who carries the del(GJB6-D13S1854) deletion in trans with Ser199Phe in GJB2. (D) GJB2 expression analysis based on the Ser199Phe sequence variant. The Ser199Phe mutation prevents AlwNI from digesting the original 201 bp fragment into a 101 and a 100 bp fragment, as illustrated in A. The digested 100 and 101 bp bands represent the wild-type allele and are undetectable in proband CE2 and almost entirely absent (the very faint remaining band is not visible on the printed picture) in proband CE3, indicating a complete lack of GJB2 expression from the del(GJB6-D13S1830) allele and an almost complete lack from the del(GJB6-D13S1854) carrying chromosome. (E) Allele-specific expression analysis for GJB2, based on Gln80Pro (239A>C). This mutation prevents Pvull-HF from digesting the 728 bp RT-PCR product in proband CE4. The digested bands represent the wild-type allele, which are almost undetectable in proband CE4 indicating a nearly complete lack of GJB2 expression from the del(GJB6-D13S1854) carrying chromosome. M = marker; ud = undigested, no restriction enzyme added; d = digested, restriction enzyme added.

doi:10.1371/journal.pone.0021665.g002

Figure 3. Allele-specific expression analysis for GJB2, based on 35delG. (A) A schematic representation (not to scale) of BstNI digestion analysis [10]. A 139 bp PCR product was amplified from GJB2 cDNA. Bars indicate GJB2 exons. The translated region within exon 2 is shaded. GJB2 mutation 35 delG is indicated (dotted arrows), as is the restriction site (arrow). The mutation eliminates a single BstNI restriction enzyme site. (B) BstNI digestion of the product amplified from proband CE5 yields an undigested 139 bp band from the 35delG allele, but the 110 bp digestion product expected from the wild-type allele is virtually absent. The wild-type control (CTR1) yields the expected 110 bp band. M = marker; ud = undigested, no restriction enzyme added; d = digested, restriction enzyme added.

doi:10.1371/journal.pone.0021665.g003
possibility for bias in PCR amplification due to differences in investigated for potential sequence changes to minimize the analyzed regions. Furthermore, all primer binding sites were the same allele. No sequence changes were detected in the was related to the GJB6 CTR1, to confirm that the allele-specific loss of promoter region in probands CE4 and CE5 as well as in control the expected transcript. Fig 3B). The control (CTR1) on the other hand clearly exhibited substantiating the almost complete down-regulation of the 110 bp fragment associated with the wild-type allele, which clearly expressed only the expected wild-type allele. The allele-specific expression assay demonstrated the 728 bp uncut transcript from the allele in cis with del(GJB6-D13S1854) is about 77 kb smaller than the ~309 kb del(GJB6-D13S1830), which includes the former. It has been assumed but not previously demonstrated that del(GJB6-D13S1854) impairs GJB2 expression in an allele-specific manner similar to what has been reported for del(GJB6-D13S1830). We determined the effect on GJB2 expression by using qualitative allele-specific RT-PCR analyses to assess the relative abundance of GJB2 transcript from both alleles. The studies were performed on readily accessible buccal epithelium, which expresses both Cx26 and Cx30 and has been postulated to contain one or more functional regulatory elements that control expression of both proteins [10,12,13]. Three probands were identified who carried del(GJB6-D13S1830) and each had different mutations in the GJB2 gene, in trans (Table 1). Their hearing phenotypes ranged from moderate to profound and were not obviously correlated with the severity of the GJB2 change (Table 1). In contrast to the previously reported and additional, currently included, individuals with the larger del(GJB6-D13S1830) in whom we could not detect any GJB2 expression from that allele (CE1 and CE2; [13]), there was reproducibly suppressed and barely detectable low residual GJB2 expression from the chromosomes which carried del(GJB6-D13S1854) (CE3, CE4 and CE5, Figs 2 and 3). The controls expressed both alleles normally, as expected (CTR2 and CTR3, Fig 2D, and CTR1, Fig 3B). This interesting difference between the two deletions supports the putative presence of multiple regulatory elements upstream of GJB6. Because the smaller deletion maintains at least some residual GJB2 expression one could assume that some such regulatory or locus-control elements are not deleted with the 77 kb shorter del(GJB6-D13S1854).

It is as yet unclear how, exactly, a lack of transcription results in a hearing loss phenotype and to what extent partial or complete deletion of the GJB6 gene contributes to this phenotype in humans. However, some such processes have been studied in some of the connexins and are clinically relevant [20]. Co-regulation of the two connexins has been explored by immunohistochemistry as well as quantitative PCR analysis in Cx30 knock-out mouse

**Figure 4. GJB2 expression of a del(GJB6-D13S1830) homozygous patient.** PCR amplification was performed after reverse transcription using primers specific for GJB2 cDNA (F1 and R1, Table 2 and Fig 2A). The wild-type control CTR1 amplified a 728 bp segment characteristic for GJB2 while del(GJB6-D13S1830) homozygous patient CE1 did not express that band at all. β-actin was used as an RNA expression control. Both samples amplified the expected 626 bp β-actin band specific for cDNA.

doi:10.1371/journal.pone.0021665.g004

different GJB2 mutations in trans. By sequence analysis of GJB2, proband CE4 was heterozygous for Gln80Pro (239A>C). The allele-specific expression assay demonstrated the 728 bp uncut band from that allele, but revealed only minimal residual expression at the wild-type fragments (CE4, Fig 2E). Thus, the GJB2 transcript from the allele in cis with del(GJB6-D13S1854) is almost entirely absent (Fig 2E). The respective control in this case was a sample from an individual without GJB2 mutations (CTR1), which clearly expressed only the expected wild-type allele. The third proband with del(GJB6-D13S1854) had moderate to severe hearing loss and was compound heterozygous with 35delG in the GJB2 gene, in trans. Again, the allele-specific assay virtually lacked the 110 bp fragment associated with the wild-type allele, substantiating the almost complete down-regulation of GJB2 expression from the chromosome with del(GJB6-D13S1854) (CE5, Fig 3B). The control (CTR1) on the other hand clearly exhibited the expected transcript.

We sequenced GJB2 exon 1, its splice sites and the proximal promoter region in probands CE4 and CE5 as well as in control CTR1, to confirm that the allele-specific loss of GJB2 expression was related to the GJB6 deletions and not to another mutation on the same allele. No sequence changes were detected in the analyzed regions. Furthermore, all primer binding sites were investigated for potential sequence changes to minimize the possibility for bias in PCR amplification due to differences in primer binding affinity. No sequence changes in primer binding sites were identified.

**Discussion**

The main purpose of this study was to demonstrate an effect on GJB2 expression by del(GJB6-D13S1854), which is internal to the larger and more prevalent del(GJB6-D13S1830) deletion, which we studied previously [13]. Secondarily, we aimed to refine the location of the GJB2 regulatory element(s) that is/are hypothesized to be within the deleted regions. Using immunostaining, a cell-specific loss of Cx26 expression was observed in the sweat glands of an individual compound heterozygous for 35delG in GJB2 and del(GJB6-D13S1830) in GJB6. The functional effect on Cx26 protein pointed to the disruption of a putative cis-regulatory element which appears to function with cell-type specificity within the sweat gland [19]. Until recently [12,13], however, no more direct support of the existence or effects of such elements was reported.

Deletion del(GJB6-D13S1834) is one of four reported DFNB1 deletions (Fig 1), of which only one (>920 kb deletion) directly affects the GJB2 gene [11]. Another pathogenic DFNB1 deletion was identified entirely upstream from GJB6 (Fig 1) in a large kindred of German extraction. This ~131 kb deletion segregated with profound deafness when in trans with 35delG in GJB2. Allele-specific expression demonstrated markedly reduced expression for GJB2 as well as GJB6, with under-representation of the allele with the deletion [10,12]. This deletion remains unique to this family [12]. del(GJB6-D13S1854) is about 77 kb smaller than the ~309 kb del(GJB6-D13S1830), which includes the former. It has been assumed but not previously demonstrated that del(GJB6-D13S1854) impairs GJB2 expression in an allele-specific manner similar to what has been reported for del(GJB6-D13S1830). We determined the effect on GJB2 expression by using qualitative allele-specific RT-PCR analyses to assess the relative abundance of GJB2 transcript from both alleles. The studies were performed on readily accessible buccal epithelium, which expresses both Cx26 and Cx30 and has been postulated to contain one or more functional regulatory elements that control expression of both proteins [10,12,13]. Three probands were identified who carried del(GJB6-D13S1834) and each had different mutations in the GJB2 gene, in trans (Table 1). Their hearing phenotypes ranged from moderate to profound and were not obviously correlated with the severity of the GJB2 change (Table 1). In contrast to the previously reported and additional, currently included, individuals with the larger del(GJB6-D13S1830) in whom we could not detect any GJB2 expression from that allele (CE1 and CE2; [13]), there was reproducibly suppressed and barely detectable low residual GJB2 expression from the chromosomes which carried del(GJB6-D13S1854) (CE3, CE4 and CE5, Figs 2 and 3). The controls expressed both alleles normally, as expected (CTR2 and CTR3, Fig 2D, and CTR1, Fig 3B). This interesting difference between the two deletions supports the putative presence of multiple regulatory elements upstream of GJB6. Because the smaller deletion maintains at least some residual GJB2 expression one could assume that some such regulatory or locus-control elements are not deleted with the 77 kb shorter del(GJB6-D13S1854).
cultures [21]. Cx26 mRNA and protein were down-regulated in non-sensory cochlear cells, which could be overcome by over-expression of Cx30 after transduction with bovine adenovirus-associated virus. The expression of Cx26 and Cx30 in mouse cultures was inter-related and reciprocal, because down-regulation of Cx30 in cultures of a mouse model with ablation of Cx26 negatively impacted the expression of Cx30 as well as the communication between cells [21].

In summary, we conclude that the hearing loss in probands with the del(GJB6-D13S1835) deletion (and a heterozygous mutation in GJB2) results from a lack of functional Cx26 protein similar to the patients hearing larger deletion del(GJB6-D13S1830) [Figs 2 and 4, [13]]. Our results are supportive of the presence of GJB2 cis-regulatory element(s) upstream GJB6 and narrow down the location of those putative element(s) that most powerfully impact GJB2 expression.

Acknowledgments

We thank Drs. Silvia Florez, Lisbeth Morales, Liliana Vertel and Greizy Lopez for their help in clinical evaluation, and laboratory research of the Colombian deaf population included in this study.

Author Contributions

Conceived and designed the experiments: JRP IS. Performed the experiments: JRP. Analyzed the data: JRP MLT NG IS. Contributed reagents/materials/analysis tools: MLT NG IS. Wrote the paper: JRP IS.

References

1. Ahmad S, Chen S, Sun J, Lin X (2003) Connexins 26 and 30 are co-assembled to form gap junctions in the cochlea of mice. Biochem Biophys Res Commun 307: 362-368.
2. Zhao H-B, Kikuchi T, Ngezahayo A, White TW (2006) Gap junctions and cochlear homeostasis. J Membr Biol 209: 177-186.
3. Kremens O, Van Naarden Braun K, Boyle C (2002) GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: A HotGE review. Genet Med 4: 238-274.
4. Hilgert N, Smith RJH, Van Camp G (2009) Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics? Mutat Res 661: 119-96.
5. Lerer I, Saqi M, Ben-Neriah Z, Wang T, Levi H, et al. (2001) A deletion mutation in GJB6 cooperating with a GJB2 mutation in trans in non-syndromic deafness: a novel founder mutation in Ashkenazi Jews. Hum Mut 18: 460-469.
6. del Castillo I, Villamar M, Moreno-Pelayo MA, del Castillo EJ, Alvarez A, et al. (2002) A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. N Engl J Med 346: 243-249.
7. Pallares-Ruiz N, Blanchet P, Mondain M, Claustres M, Roux A (2002) A large deletion including most of GJB6 in recessive non syndromic deafness: a digenic effect? Eur J Hum Genet 10: 72-76.
8. del Castillo I, Moreno-Pelayo MA, del Castillo EJ, Brownstein Z, Marlin S, et al. (2003) Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. Am J Hum Genet 73: 1452-1458.
9. del Castillo EJ, Rodriguez-Ballesteros M, Alvarez A, Hutchin T, Leonard E, et al. (2005) A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment. J Med Genet 42: 589-594.
10. Wilch E, Zha M, Burkhart KB, Regier M, Elfenbein JL, et al. (2006) Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele. Am J Hum Genet 79: 174-179.
11. Feldmann D, Le Maréchal C, Jonard L, Thierry P, Cazaja C, et al. (2009) A new large deletion in the DFNB1 locus causes nonsyndromic hearing loss. Eur J Med Genet 52: 195-200.
12. Wilch E, Azaiez H, Fisher RA, Elfenbein J, Murgia A, et al. (2010) A novel DFNB1 deletion allele supports the existence of a distant cis-regulatory region that controls GJB2 and GJB6 expression. Clin Genet 78: 267-274.
13. Rodríguez-París J, Schrijver I (2009) The digenic hypothesis unraveled: the GJB6 del(GJB6-D13S1830) mutation causes allele-specific loss of GJB2 expression in cis. Biochem Biophys Res Commun 389: 554-559.
14. Putcha GV, Begiani BA, Bloo S, Booker JK, Carey JC, et al. (2007) A multicenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. Genet Med 9: 415-426.
15. Mueller RF, Nehammer A, Middleton A, Houseman M, Taylor GR, et al. (1999) Congenital non-syndromal sensorineural hearing impairment due to connexin 26 gene mutations – molecular and audiological findings. Int J Pediatr Otorhinolaryngol 50: 3-11.
16. Raff T, van der Giet M, Wiederholt T, Paul M (1997) Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. Biotechniques 23: 456-460.
17. Wilcox SA, Oshorn AH, Dahl HHM (2000) A simple PCR test to detect the common 35delG mutation in the connexin 26 gene. Mol Dig 5: 75-78.
18. Matos TD, Caria H, Simões-Teixeira H, Aasen T, Nickel R, et al. (2007) A novel hearing-loss-related mutation occurring in the GJB2 basal promoter. J Med Genet 44: 721-725.
19. Common JEA, Binzer-Glindzicz M, O’Toole EA, Barnes MR, Jenkins L, et al. (2003) Specific loss of connexin 26 expression in ductal sweat gland epithelium associated with the deletion mutation del(GJB6-D13S1830). Clin Exp Dermatol 28: 681-693.
20. Vinken M, De Rop E, Decrock E, De Vuyt E, Leybaert L, et al. (2009) Epigenetic regulation of gap junction intercellular communication: more than a way to keep cells quiet? Biochim Biophys Acta 1795: 53-61.
21. Orlotano S, Di Pasquale G, Crispino G, Anselmi F, Mammano F, et al. (2008) Coordinated control of connexin 26 and connexin 30 at the regulatory and functional level in the inner ear. Proc Natl Acad Sci USA 105: 18776-18781.