A NOVEL DFNB1 DELETION ALLELE SUPPORTS THE EXISTENCE OF A DISTANT CIS-REGULATORY REGION THAT CONTROLS GJB2 AND GJB6 EXPRESSION

Ellen Wilch,
Genetics Program, Michigan State University, East Lansing MI 49224 USA

Hela Azaiez,
Molecular Otolaryngology Research Laboratories, University of Iowa, Iowa City, IA 52242 USA

Rachel A. Fisher,
Department of Pediatrics, Michigan State University, East Lansing, MI 48824 USA

Jill Elfenbein,
Department of Communicative Sciences and Disorders, Michigan State University, East Lansing, MI 48824 USA

Alessandra Murgia,
Department of Pediatrics, University of Padua, 35128 Padua, Italy

Ralf Birkenhäger,
Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University Medical Center Freiburg, D-79106 Freiburg, Germany

Hanno Bolz,
Institute of Human Genetics, University of Cologne, Cologne 50931, Germany

Sueli Matilde Silva Costa,
Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Sao Paolo, Brazil

Ignacio del Castillo,
1 Unidad de Genética Molecular, Hospital Universitario Ramón y Cajal, Madrid, Spain 2 Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain

Thomas Haaf,
Institute of Human Genetics, Johannes Gutenberg University Mainz, 55131 Mainz, Germany

Lies Hoefsloot,
Nijmegen Centre of Molecular Life Sciences, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, 6500 HB Nijmegen, The Netherlands

Hannie Kremer,
Dept. Otorhinolaryngology, Radboud University Nijmegen Medical Centre, & Nijmegen Centre of Molecular Life Sciences, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, 6500 HB Nijmegen, The Netherlands

Christian Kubisch,
Institute of Human Genetics, Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany

Cedric Le Marechal,
Université Européenne de Bretagne & Université de Brest ; INSERM U613 ; Centre Hospitalier Universitaire (CHU) de Brest, Brest, France

Arti Pandya,
Department of Human and Molecular Genetics, Virginia Commonwealth University, Richmond, VA 23298-0033 USA

Edi Lúcia Sartorato,
Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Sao Paolo, Brazil

Eberhard Schneider,
Institute of Human Genetics, Johannes Gutenberg University Mainz, 55131 Mainz, Germany

Guy Van Camp,
Department of Medical Genetics, University and University Hospital of Antwerp, Antwerp, Belgium

Wim Wuyts,
Department of Medical Genetics, University and University Hospital of Antwerp, Antwerp, Belgium

Richard HJ Smith, and
Molecular Otolaryngology Research Laboratories and Department of Otolaryngology-Head and Neck Surgery, University of Iowa Hospitals and Clinics, Iowa City, IA 52242 USA

Karen H. Friderici
Department of Microbiology and Molecular Genetics and Department of Pediatrics, Michigan State University, East Lansing, MI 48824 USA

Abstract

Eleven affected members of a large German-American family segregating recessively inherited, congenital, non-syndromic sensorineural hearing loss (SNHL) were found to be homozygous for the common 35delG mutation of GJB2, the gene encoding the gap junction protein Connexin 26. Surprisingly, four additional family members with bilateral profound SNHL carried only a single 35delG mutation. Previously, we demonstrated reduced expression of both GJB2 and GJB6 mRNA from the allele carried in trans with that bearing the 35delG mutation in these four persons. Using array comparative genome hybridization (arrayCGH), we have now identified on this allele a deletion of 131.4 kb whose proximal breakpoint lies more than 100 kb upstream of the transcriptional start sites of GJB2 and GJB6. This deletion, del(chr13:19,837,344-19,968,698), segregates as a completely penetrant DFNB1 allele in this family. It is not present in 528 persons with SNHL and monoallelic mutation of GJB2 or GJB6, nor have we identified any other candidate pathogenic copy number variation by arrayCGH in a subset of 10 such persons. Characterization of distant GJB2/GJB6 cis-regulatory regions evidenced by this allele may be required to find the ‘missing’ DFNB1 mutations that are believed to exist.

Keywords

Connexin 26; connexin 30; DFNB1; gene expression regulation; GJB2; GJB6; sensorineural hearing loss; sequence deletion
INTRODUCTION

Mutation in the Connexin 26 (Cx26) gap junction protein-encoding gene GJB2 (MIM 121011) is the most commonly identified cause of congenital, recessively inherited, sensorineural nonsyndromic hearing loss (DFNB1A [MIM 220290])(12). In the inner ear, Cx26 and Cx30 (encoded by GJB6 [MIM 604418], which lies 30 kb telomeric to GJB2 on human chromosome 13) are expressed in non-sensory cells of the organ of Corti and in cells of the spiral ligament and stria vascularis. Cx26/Cx30 cochlear gap junctions have been implicated in maintenance of K⁺ homeostasis in the inner ear(3-4). However, more recent studies point to a complex suite of functions for gap-junctional networks throughout the cochlea involving participation of second-messengers and including generation of the endocochlear potential(5-7). This intricate set of roles depends on a varied distribution and composition of connexon hemichannels and gap junctions throughout the epithelial supporting cell and connective tissue gap-junctional networks of the cochlea(8-12), therefore requiring tight regulation of expression of GJB2 and GJB6.

Although more than 200 mutations of GJB2, two deletions involving GJB6 (DFNB1B [MIM 612645]), and one deletion encompassing both GJB2 and GJB6 constitute the reported set of DFNB1 mutations(1,13-15), population screening commonly yields an excess of individuals with hearing loss who carry only a single identified GJB2 mutation or DFNB1 deletion(16). This finding strongly suggests that additional mutations that lie outside of the proximal promoter and transcribed regions of GJB2 remain to be identified. Here we identify a novel 131.4 kb deletion, distant from the transcriptional start sites of both GJB2 and GJB6, that segregates as a DFNB1 allele in the extended family in which it is found, and that also segregates with reduced expression of either GJB2 or GJB6 mRNA in four family members assayed. This finding has relevance for the identification of distant GJB2 and GJB6 cis-regulatory elements, as sequence variations within these elements may prove to constitute the bulk of ‘missing’ GJB2 mutations in DFNB1 hearing loss.

MATERIALS AND METHODS

Human subjects

Participating MSU-DF5 subjects were ascertained through the Oyer Speech-Language-Hearing Clinic within the Department of Communicative Sciences and Disorders at Michigan State University. Audiologic examination of family members included otoscopy, tympanometry, and pure-tone and air- and bone-conduction thresholds. DNA was isolated from blood, saliva, or cheek swabs. Genealogical information was collated from family histories and from publicly available records. Additional subjects were ascertained from hearing loss referrals to the Molecular Otolaryngology Research Laboratories at the University of Iowa. Written informed consent for genetic and audiological testing was obtained from all participants or from the parents of minors. The Michigan State University Institutional Review Board and the University of Iowa Human Subjects Committee approved all procedures.

Array Comparative Genome Hybridization

To examine potential cis-regulatory regions for mutation, we looked first for copy number variants by array comparative genome hybridization (arrayCGH) using a finely-tiled CGH microarray designed and manufactured by Nimblegen Systems. Included on the array were 384,000 overlapping oligomeric probes of 45-80 nucleotides in length from regions of non-repetitive sequence on human chromosome 13q11-12, spanning ~6.5 Mb from the most centromeric annotated sequence at chr13:18,000,000 to chr13:24,500,000 (Build 36.1). We provided Nimblegen with DNA from MSU-DF5-20, one of the four deaf family members.
with monoallelic mutation of GJB2. Nimblegen carried out the labeling and hybridizations on MSU-DF5-20 as well as on DNA from 10 additional unrelated GJB2 heterozygotes with severe-to-profound NSHL. Identification of the breakpoints of del(chr.13:19,837,344-19,968,698) was done by bidirectional Sanger sequencing of the 628 bp PCR product amplified with primers flanking the region of copy number loss (see next section). Confirmation of regions of deletion and duplication in the additional GJB2 heterozygotes and screening in 160 CEPH controls was done by PCR with flanking primers; these products were not sequenced for breakpoint identification.

Deletion screening by PCR assay

To screen for del(chr.13:19,837,344-19,968,698), we designed a 2-product multiplex PCR assay. Two primers (5′-TGGGACCAGGTCTGTTGTT-3′ and 5′-ATTGCGACTTGCTTTTCGTT) flank the deletion breakpoint and amplify a 628 bp product from genomic DNA bearing the deletion; the second primer pair (5′-GCAGCCATCTCATGGTTCT-3′ and 5′-CCAACACACAATTGGGTCACTCT-3′) amplifies an 836 bp product from sequence within the deleted interval. In a 20 ul reaction containing 1.5 mM MgCl$_2$, 25 uM primer, 0.2 mM dNTP, and 0.5 U Taq polymerase, genomic DNA (10-40 ng) was denatured for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. Products were resolved on a 1% agarose gel containing 0.3 ug/ml ethidium bromide. DNA from 220 MSU-DF5 family members, 160 CEPH controls, and 528 patients with hearing loss who are heterozygous for a known GJB2/GJB6 mutation was assayed.

RESULTS

Identification of del(chr13:19,837,344-19,968,698) in an extended family segregating DFNB1 hearing loss

Figure 1 shows a small portion of the pedigree of MSU-DF5, an extended family descended from a founder community of 500 German-Catholic emigrant families who settled in Michigan in the 19th century. Eleven living family members with hearing loss are known to be homozygous for the common European founder mutation of GJB2, 35delG (only one of those individuals is included in Fig. 1). Unexpectedly, four other family members with bilateral profound SNHL carry only a single 35delG mutation but share the same haplotype on their non-35delG chromosome. Previously, we documented allele-specific reduction of GJB2 and GJB6 expression from this allele in buccal cells from four heterozygous individuals (two deaf, with 35delG in trans; two hearing, with normal GJB2 and GJB6 alleles in trans)(17). Sequencing of the coding regions, 5′ and 3′ UTRs, proximal promoter regions, and splice sites of both GJB2 and GJB6, as well as several kilobases of sequence upstream of GJB2 failed to identify any other sequence variant unique to this low-expression allele.

A single copy region identified by arrayCGH (Fig. 2A) was confirmed by PCR and sequencing (Fig. 2B) to be a deletion of 131.4 kb that is carried in trans with the 35delG mutation in the four deaf 35delG heterozygotes. The proximal breakpoint of this deletion is in intergenic sequence at chr13:19,837,344 within a simple repeat of (TG)$_{21}$, leaving 9 TG dinucleotides intact on the deleted chromosome. The distal breakpoint occurs at chr13:19,968,698 within a LINE element located in the second intron of CRY1. The sequence flanking the breakpoints does not clearly indicate a mechanism by which the deletion was generated. No homology exists between the sequences including and immediately adjacent to the breakpoints. The existence of only a single base pair of microhomology at the breakpoint junction is consistent with nonhomologous end joining (NHEJ) and double-strand break (DSB) repair.
Del(chr13:19,837,344-19,968,698) segregates as a completely penetrant DFNB1 allele and is >300 years old

Of the 220 MSU-DF5 family members tested, 27 are heterozygous and none are homozygous for the deletion. The deletion is present on the chromosomes from which reduced expression of GJB2 and GJB6 message was documented (in MSU-DF5-20, -65, -67, and -70), and in all four deaf 35delG heterozygotes, indicating that the deletion-bearing chromosome segregates in this pedigree as a completely penetrant recessive DFNB1 allele (Figure 1). This deletion was not identified in 160 CEPH controls.

Under the assumption of identity-by-descent, and by choosing the least number of meioses to relate carriers, the deletion chromosome can be traced back to four individuals born in Germany between 1702 and 1716, all equally likely to have contributed this allele to the MSU-DF5 pedigree. Although the deletion allele is thus at least 300 years old and may exist outside of this pedigree, it has not been identified among 528 persons from the United States, Brazil, Iran, and several European countries with hearing loss who are heterozygous for a single GJB2/GJB6 mutation (Table 1).

Assessment of 35delG-heterozygotes with SNHL for incidence of pathogenic copy number variation across the DFNB1 locus

To determine if deletions may constitute a frequent mechanism for DFNB1 deafness, we performed arrayCGH on DNA from 10 unrelated 35delG-heterozygous patients with severe to profound SNHL. We identified copy number variants (CNVs) across the region (Table 2), including 5 deletions ranging in size from 130 bp to 450 bp, and 3 duplications between 65 bp and 960 bp in size. Three regions were associated with either duplication or deletion. CNVs were confirmed by PCR with flanking primers, although the breakpoints were not precisely identified. All of these CNVs were present in 160 CEPH controls at frequencies higher than 20%.

DISCUSSION

Of the nearly 100 loci mapped for human inherited nonsyndromic hearing loss since the early 1990s, 46 genes have been identified with sufficient evidence to establish causation. Mutations in two of these genes, GJB2 and SLC26A4, cause a significant proportion of hearing loss globally, with loss-of-function mutations of GJB2 estimated to be responsible for more than half of all recessively inherited NSHL in developed countries (1). This knowledge has changed the medical evaluation of families segregating presumed autosomal recessive NSHL and has made accurate genetic counseling, recurrence chance estimation, phenotype-genotype correlations and prognosis for progression of hearing loss possible when two mutant alleles of GJB2 are identified. It is widely accepted that unidentified mutations of GJB2 exist that are cis-regulatory in nature. Deletion alleles that abrogate expression of GJB2 without disrupting the gene sequence are important for identifying candidate cis-regulatory regions for mutation analysis.

The deletion we have described, del(chr13:19,837,344-19,968,698), represents the fourth DFNB1 deletion allele, and the third in which GJB2 is left intact. Del(GJB6-D13S1830)(13'18'19) and del(GJB6-D13S1854)(14), deletions of 309 kb and 232 kb, respectively, truncate GJB6 and extend telomerically (Figure 2C). Lerer et al(18) showed GJB2 expression from both alleles in lymphocytes from a patient heterozygous for del(GJB6-D13S1830) and on this basis suggested that a digenic interaction between GJB2 and GJB6 may be responsible for the hearing loss in these individuals. Our allele-specific expression assay results on del(chr13:19,837,344-19,968,698), which suggest otherwise, were consistently replicated using stratified epithelial cell-derived cDNA from buccal swabs while assays of
lymphocyte-derived cDNA from the same individuals gave inconsistent results (data not shown). This discrepancy may indicate that regulation of GJB2 and GJB6 differs with tissue type, and that expression assayed in lymphocytes may not be relevant to expression in cochlear cells.

Common et al(20) also demonstrated loss of normal Cx26 expression from the deletion-bearing allele in stratified epithelial skin cells in an individual with hearing loss and a 35delG/del(GJB6-D13S1830) genotype. These results are consistent with hypotheses of complex cis-regulation of GJB2 and loss of an important GJB2 regulatory element as a result of these deletions. Qualitative loss of GJB2 expression from alleles bearing del(GJB6-D13S1830) in compound heterozygosity with GJB2 mutations/variants E47X, M34T, and V27I/E114G has since been demonstrated in three individuals(21), further supporting this mechanism of action.

Feldmann et al(15) have provided additional evidence against the GJB2/GJB6 digenic hypothesis. They have documented a >920 kb deletion encompassing GJB2, GJB6, GJA3 and several other genes that is present in trans with the V84M mutation of GJB2 in a deaf individual. Three other persons with normal hearing in this pedigree carry this contiguous gene deletion, indicating that loss of single copies of GJB2 and GJB6, per se, cannot explain the pathogenicity of the two GJB6-truncating deletion alleles, del(GJB6-D13S1830) and del(GJB6-D13S1854).

Del(chr13:19,837,344-19,968,698) is of particular interest as it leaves both GJB2 and GJB6 completely intact, has a proximal breakpoint substantially farther from GJB2 than the GJB6-truncating DFNB1 deletion alleles, and segregates with reduced expression of both GJB2 and GJB6. These findings suggest that time- and tissue-specific enhancer elements for both genes may lie a considerable distance upstream, and/or that a locus control region exists for these two genes. We hypothesize a similar mechanism of pathogenesis for all three deletion alleles that involves, in addition to loss or reduction in expression of GJB6, the loss of a critical cis-regulatory element for GJB2 located within the common 95.4 kb genomic interval that is deleted in all three alleles. Sequence conservation between species is one metric by which candidate noncoding regulatory DNA may be identified. Human-mouse sequence conservation and rank VISTA (rVISTA) calculations of potential regulatory function(22-23) (Figure 2D, reproduced from the VISTA browser) show numerous candidate regulatory elements within this interval.

Since we found del(chr13:19,837,344-19,968,698) in only one extended family, as an alternate, less parsimonious hypothesis, del(chr13:19,837,344-19,968,698) could be in linkage disequilibrium with a pathogenic single nucleotide substitution or other undetected DNA lesion closer to both GJB2 and GJB6 that disrupts cis-regulatory function of both genes coordinately. This possibility also permits a common mechanism of pathogenesis of the three deletion alleles which leave GJB2 intact, and accommodates the evidence of loss of GJB2/Cx26 expression observed from the del(GJB6/D13S1830) allele by Rodrigues-Paris et al(21) and by Common et al(20).

The identification of additional deletions is possible with the increasing accessibility of arrayCGH and other techniques to assess CNV. Although patients with severe-to-profound hearing loss and monoallelic GJB2 mutation are candidates for pathogenic CNV, our findings from 10 such patients suggest that while CNV is common, identifying the regulatory regions may require an alternative approach such as enhancer-reporter assays. The CNVs we found among our 10 patients were all found among CEPH controls at frequencies higher than 20%.
Sequence variation within regulatory elements can give rise to allele-specific quantitative differences in expression of message that may yield phenotypic variation(24–25). Variability in degree of hearing loss is a well-documented feature of GJB2 deafness, even among persons with biallelic null (protein-truncating) mutations that completely abolish Cx26 protein expression(16–26). No modifier genes have yet been identified to explain this phenotypic variability(27). We hypothesize that sequence variation in cis that regulates GJB6 expression from a distance may be partially responsible for some of this variability.

In summary, our studies of del(chr13:19,837,344-19,968,698) support the presence of distant cis-regulation of GJB2 and substantially reduce the span of chromosome 13 that is most strongly implicated in this function. We also provide the first evidence of distant cis-regulation of GJB6. Additional efforts to elucidate and characterize distant GJB2 and GJB6 cis-regulatory regions are clearly warranted.

Acknowledgments

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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Array CGH</td>
<td>array comparative genome hybridization</td>
</tr>
<tr>
<td>CEPH</td>
<td>Centre d’Etude du Polymorphisme Humain</td>
</tr>
<tr>
<td>CNV</td>
<td>copy number variant</td>
</tr>
<tr>
<td>CX26</td>
<td>Connexin 26</td>
</tr>
<tr>
<td>CX30</td>
<td>Connexin 30</td>
</tr>
<tr>
<td>DSB</td>
<td>double-strand break</td>
</tr>
<tr>
<td>LINE</td>
<td>long interspersed nuclear element</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SNHL</td>
<td>sensorineural hearing loss</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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</table>

REFERENCES


Figure 1.
The MSU-DF5 sub-pedigree shown here includes the four profoundly deaf family members who are compound heterozygotes for del(chr13:19,837,344-19,968,698) (green shading) and for the 35delG mutation of \textit{GJB2} (blue shading) which segregates within this family. Of the 220 family members screened, twenty-seven unaffected persons are heterozygous for the deletion and are negative for 35delG; they are shown here, although a number of unaffected siblings who are negative for both mutations are not. The most recent common ancestors of the known carriers of del(chr13:19,837,344-19,968,698) were born in the early eighteenth century in northern Europe. We previously documented reduced expression of \textit{GJB2} in three persons (red arrows) and \textit{GJB6} in one person (yellow arrow) from the allele now known to bear del(chr13:19,837,344-19,968,698).
Figure 2.
Characterization of novel DFNB1 deletion allele del(chr13:19,837,344-19,968,698). (A) Custom arrayCGH across a 6.5 Mb region of human chromosome 13 indicates a region of single copy number loss in MSU-DF5-20.
(B) Del(chr13:19,837,344-19,968,698) breakpoint junctional sequence amplified from MSU-DF5-20 DNA and aligned with human genome reference sequence (Build 36.1) shows a single basepair of microhomology at the breakpoint.
(C) Del(chr13:19,837,344-19,968,698) overlaps with DFNB1 deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) over a 95.4 kb interval upstream of the transcriptional...
start sites (indicated by right-angled arrows) of GJB2 and GJB6. CRYLI is also shown. Exons of all genes are indicated by vertical lines.

(D) Upper VISTA track shows sequence conservation between human (March 2006) and mouse (July 2007) genomes over the 95.4 kb interval common deletion interval indicated in Fig. 2C; pink-colored peaks represent regions of 100+ bp of non-coding sequences conserved at ≥70% identity. Lower track shows rankVISTA (rVISTA) predictions (−log_{10}[P-value]) of potential regulatory function based on multispecies sequence conservation combined with identification of transcription factor binding sites (http://genome.lbl.gov/vista/index.shtml).
### Table 1
Number of affected individuals with monoallelic \( GJB2/GJB6 \) mutation screened for \( \text{del(chr13:19,837,344-19,968,698)} \).

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<th>Laboratory (Investigators)</th>
<th>N</th>
<th>Reported ethnicity or nationality</th>
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<td>USA/Iowa (Smith/Azaiez)</td>
<td>55</td>
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<tr>
<td>USA/Virginia (Pandya)</td>
<td>132</td>
<td>96 caucasian, 4 black, 25 hispanic, 7 other</td>
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<tr>
<td>Spain (del Castillo)</td>
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<tr>
<td>Brazil (Sartorato/da Silva Costa)</td>
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<tr>
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<tr>
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<td>31</td>
<td>23 German, 5 Turkish, 2 Moroccan, 1 Spanish</td>
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<tr>
<td>Italy (Margia)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Belgium (Van Camp/Wuyts)</td>
<td>58</td>
<td>40 Belgian, 18 Iranian</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>528</td>
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</tr>
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### Table 2

**List of CNVs in the DFNB1 region**

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<thead>
<tr>
<th>Position</th>
<th>Variation Length</th>
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<tbody>
<tr>
<td>Chr13:19686728-19686792</td>
<td>dup</td>
<td>65</td>
</tr>
<tr>
<td>Chr13:19691120-19691940</td>
<td>dup</td>
<td>820</td>
</tr>
<tr>
<td>Chr13:19722020-19722070</td>
<td>del/dup</td>
<td>50</td>
</tr>
<tr>
<td>Chr13:19749870-19750200</td>
<td>del</td>
<td>340</td>
</tr>
<tr>
<td>Chr13:19768000-19768130</td>
<td>del</td>
<td>130</td>
</tr>
<tr>
<td>Chr13:199818780-199819200</td>
<td>del</td>
<td>420</td>
</tr>
<tr>
<td>Chr13:19835210-19835420</td>
<td>del/dup</td>
<td>200</td>
</tr>
<tr>
<td>Chr13:19863060-19864020</td>
<td>del/dup</td>
<td>960</td>
</tr>
<tr>
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<td>del</td>
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</tr>
<tr>
<td>Chr13:19969740-19970700</td>
<td>dup</td>
<td>960</td>
</tr>
<tr>
<td>Chr13:19974330-19974480</td>
<td>del</td>
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* Length and position are approximate; del: deletion; dup: duplication