Advanced genome-wide screening in human genomic disorders

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Chapter 8

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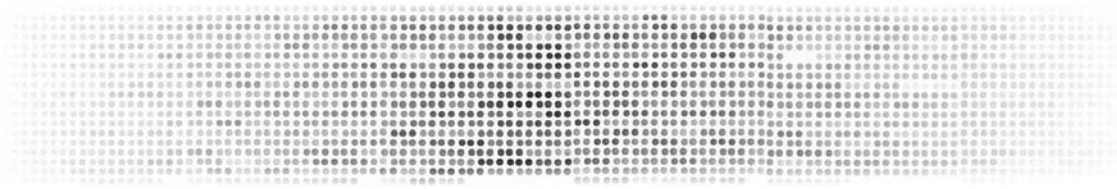

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A homozygous deletion of a normal variation locus causes syndromic hearing loss in a patient from non-consanguineous parents

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ABSTRACT

Background:
International databases with information on copy number variation of the human genome are an important reference for laboratories working with high resolution whole genome screening platforms. Genomic deletions or duplications which have been detected in the healthy population and thus marked as normal copy number variants (CNVs) can be filtered out using these databases when searching for pathogenic copy number changes in patients. However, a potential pitfall of this strategy is that reported normal CNVs often do not elicit further investigation, and thus may remain unrecognized when they are present in a (pathogenic) homozygous state. The impact on disease of CNVs in the homozygous state may thus remain undetected and underestimated.
Methods and results:
In a patient with syndromic hearing loss, array comparative genomic hybridization (array-CGH) and multiple ligation-dependent probe amplification (MLPA) revealed a homozygous deletion on 15q15.3 of a normal variation region, inherited from hemizygous carrier parents. The deletion is about 90 kilobases and contains four genes including the *STRC* gene, which is involved in autosomal recessive deafness (DFNB16). By screening healthy control individuals we estimated the frequency of hemizygous deletion carriers to be about 1.6%
Conclusion:
A homozygous deletion of a CNV region causing syndromic hearing loss was characterized by a panel of molecular tools. Together with the estimated frequency of the hemizygous deletion these results underline the potential clinical relevance of the 15q15.3 locus for patients with (syndromic) hearing impairment. This example shows the importance of not automatically eliminating registered CNVs from further analysis.

INTRODUCTION

Since the implementation of techniques for the detection of copy number variations of the human genome, such as array comparative genomic hybridization (array-CGH), single nucleotide polymorphism (SNP-) or oligo-arrays and paired-end mapping, it has become evident that copy number variations (CNVs) contribute for a large part to the total genetic variation.[1-12] The majority of this data is being pooled now in databases such as the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/).[8,13] Many of these CNV regions harbor genes. Wong et al.[2] found that 1673 RefSeq-genes overlapped 546 of 800 found CNVs. In the study of Redon et al.[1] 2908 RefSeq-genes were found in a total of 1447 CNV regions. The function of the genes located in CNV regions are often not related to primary development, but are in general more related to sensory perception.[1,2] In this way, they probably contribute to normal population variation.

The increased resolution of genome wide molecular karyotyping tools has led to the daunting task of classifying CNVs as pathogenic or non-pathogenic in the clinical setting. To facilitate the interpretation of detected copy number alterations, genome browsers and linked databases such as the DECIPHER database (http://www.sanger.ac.uk/PostGenomics/decipher/)[14] and the DGV database are widely used. The DGV database is often used as a reference template to quickly filter benign variants out of the substantial amount of CNVs found in screening patients. Similarly, the inheritance status of CNVs is used to determine whether they may be pathogenic, based on the assumption that copy number alterations inherited from a healthy parent are less likely to be disease-related or have phenotypic consequences.[15] However, this procedure of selection must be done prudently, as important information may be too easily disregarded.

In the recently described deafness-infertility syndrome (DIS) [MIM #611102] hearing loss and infertility are caused by a recessive contiguous gene deletion on chromosome 15q15.3.[16,17] The region consists of a segmental duplication, with four known active genes in the proximal region: the cation channel *CATSPER2* gene [MIM *607249], which has a function in sperm motility[16], *STRC,* coding for stereocilin [MIM *606440], which is expressed in the sensory areas of the inner ear[18], the inositol phosphate kinase *HISPPD2A* [MIM *610979] and creatine mitochondrial kinase-1A (*CKMT1A*). Homozygous mutations in *STRC* have been described in non-syndromic autosomal recessive sensorineural hearing loss linked to the DFNB16 locus [MIM #603720].[16,18] Only CKMT1A has a functional homologue in the duplicated region, named CKMT1B, while all others are pseudogenes with inactivating mutations. Recombination of the segmental duplication on 15q15.3, possibly mediated by non-allelic homologous recombination (NAHR), may lead to loss or gain of the repeats.

By using different molecular genetic screening tools we ascertained a patient with a homozygous deletion of the proximal repeat region of 15q15.3, containing the active genes. Detailed characterization of the deletion proved that the deletion was inherited from non-consanguineous parents who appear to be heterozygous carriers. To further assess the nature and frequency of the

rearrangements in this area, a control panel from the normal hearing population, a panel of patients with sensorineural hearing loss and the HapMap samples with reported variation in this region and their available family members (according to the DGV) were tested with the multiplex ligation dependent probe amplification (MLPA) set developed for this genomic segmental duplication region on chromosome 15q15.3. The HapMap[19] samples are a set of cell lines from healthy volunteers from various ethnic backgrounds, used to study human variation. Here we prove that a reported normal variant can have serious phenotypic consequences in a recessive fashion which emphasizes the need for careful interpretation of copy number variation data.

METHODS

The patient described here is a 10-year-old boy from non-consanguineous, healthy parents. He has slowly progressive bilateral sensorineural hearing loss, disharmonious mental retardation (IQ 56), short stature and dysmorphic features. Dysmorphic features include small palpebral fissures, synophrys, a high nasal bridge, brachydactyly and puffy hands and feet. The audiogram of the patient at the age of 7 years shows a moderate hearing impairment of all frequencies (fig 1). Informed consent was obtained from the parents and the proband.

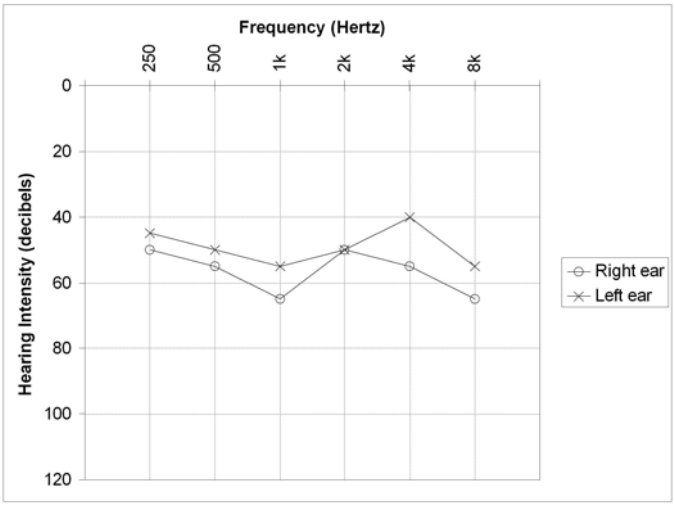

Figure 1. Audiogram of the proband showing moderate bilateral hearing loss.

Routine GTG-banding of cultured lymphocytes was performed using standard cytogenetic analysis. DNA was isolated from whole blood using standard isolation techniques. Routine amplification and sequencing for gap junction protein, beta-2 (*GJB2*) [MIM *121011] coding mutations was performed as described previously.[20]
Array-CGH was performed using ~1.0Mb spaced whole genome large insert clone arrays, which were made available by the Wellcome Trust Sanger Institute, according to published methods.[21-23]

A HumanHap300-duo BeadChip SNP-array experiment (Illumina B.V., Eindhoven, the Netherlands) was performed according to procedures as suggested by the manufacturer. The SNP array, consisting of 317,000 oligonucleotide probes, was analyzed with the BeadStudio v3.0 software provided.
MLPA probes were designed to delineate the deletion further, because the coverage of the SNP platform in this 15q15.3 region was poor. MLPA was used to confirm the results, determine the extent of the deletions, and further analyze this region in other patient groups. Despite the fact that there is over 98% homology between the segmental duplicons, for probe design it was possible to distinguish between the proximal and distal repeat by using the small regions of non-homology or paralogous sequence variants (PSVs), known from the reference sequence. This resulted in a set of 23 probes specific for the active proximal and the inactive (containing pseudogenes) distal region, with a resolution of approximately 10 kb, located between 41.63 Mb and 41.83 Mb on chromosome 15q15.3. Three probes were localized within the active *STRC* gene. Probes were ordered from Operon (Cologne, Germany). The sequences are available in the online supplemental table 1. MLPA was performed as described previously,[24] with the adaptation that the ligation was performed at an annealing temperature of 60°C.
Long-range PCR and sequencing reactions were used characterize the breakpoints of the maternally and paternally derived deletions in the patient. PCR primers were designed at PSVs region according to the reference sequence, between the location of the MLPA probes that showed a normal ratio and a homozygous deletion. Theoretically, fragments from both the distal and the proximal repeat could be amplified with these primers. PCR and subsequent sequencing was performed with the PCR primers on the patient and both parents. The sequences were aligned to the human reference sequence, NCBI Build 36.1, using the online BLAT tool of the UCSC genome browser (http://genome.ucsc.edu/).[25]

RESULTS

Routine GTG-banding revealed a normal male karyotype (46,XY). Routine screening for mutations in *GJB2*, the most common pathogenic gene in hearing loss, revealed no mutations in the coding sequence.
Analysis of genomic DNA of the patient on ~1Mb spaced large clone insert array-CGH revealed two single clone deletions on chromosome 15. One deletion was found in band 15q26.2, involving bacterial artificial chromosome (BAC) clone RP11-315L6. When mapping this clone back to a genome browser and the DGV, this clone localized to a complete gene desert region which is described to be involved in normal variation. The other deletion found on chromosome 15 involved BAC clone RP11-263I19 (fig 2A) located at 15q15.3. Although this BAC clone is located in an area also previously reported to be involved in normal variation[1,9,10,12], this region has also been described to be involved in sensorineural hearing loss[18] as well as the recently published deafness-infertility syndrome.[16,17]

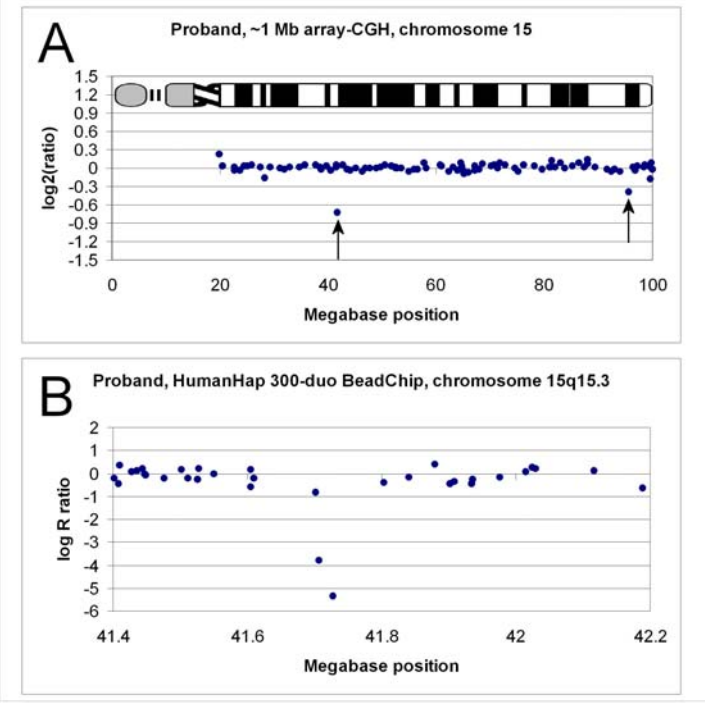

Figure 2. Micro-array profiles. (A) Large-insert clone array-CGH profile of the proband of chromosome 15 showing the deletion of BAC RP11-263I19 located around 41.7 Mb and BAC RP11-315L6 around 95.6 Mb (indicated by the black arrows). (B) SNP array profile of chromosome 15q15.3 of the proband showing the deletion of SNPs rs2927071 and rs8042868 corresponding to RP11-263I19 region.

To further characterize this deletion, a HumanHap300-duo BeadChip SNP-array experiment was performed. The results ruled out any unknown consanguinity of the parents or uniparental disomy in the patient, since the SNP profiles did not show large blocks of homozygous alleles in the patient. The hybridization showed a homozygous loss of SNPs rs2927071, rs8042868 and rs8038068, which were all located in a stretch of 25 kb, inside the proximal repeat sequence containing the four active genes (fig 2B). The deletion at 15q26.2 was also detected on the Illumina platform and was in size and location exactly comparable to the reported copy number variant (CNV) reported by Redon et al.[1]
Because of poor coverage of the SNP platform in this particular area, MLPA probes were designed to delineate the deletion further. The proband showed a homozygous deletion from 41.66 to 41.74 Mb. The deletions on both alleles involve the majority of the proximal repeat region, deleting or disrupting the active genes. The deletion size was at least 80 kb and a matching heterozygous deletion was found in both parents (fig 3).
Figure 4 shows the sequencing result of a conclusive PSV between the MLPA probes where breakpoints on the paternal and maternal allele had to have taken place. This sequencing result revealed proof of one heterozygous call in the patient and proved that the sequence of the maternally derived allele in the patient only consisted of the distal repeat and the paternally derived allele only

of the proximal repeat (fig 4). Sequencing of other regions containing PSVs with regards to breakpoint mapping was inconclusive.
To assess the frequency and the type of alteration of this locus, the developed MLPA set was tested on a panel of 64 normal hearing persons and 20 in-house control samples. Also with the same MLPA panel, a group of 45 patients with non-syndromic sensorineural hearing loss was screened for genetic alterations. This group of patients was proven by sequencing to have no mutation in the *GJB2* gene.[20] The MLPA test revealed that none of these samples had a contiguous deletion the size of the duplicon region. One sample in the patient group and two in the control group were found to have a duplication of the proximal repeat part, with a size comparable to the size of the deletion of the patient described herein, and the previously published consanguineous families.[16,17]

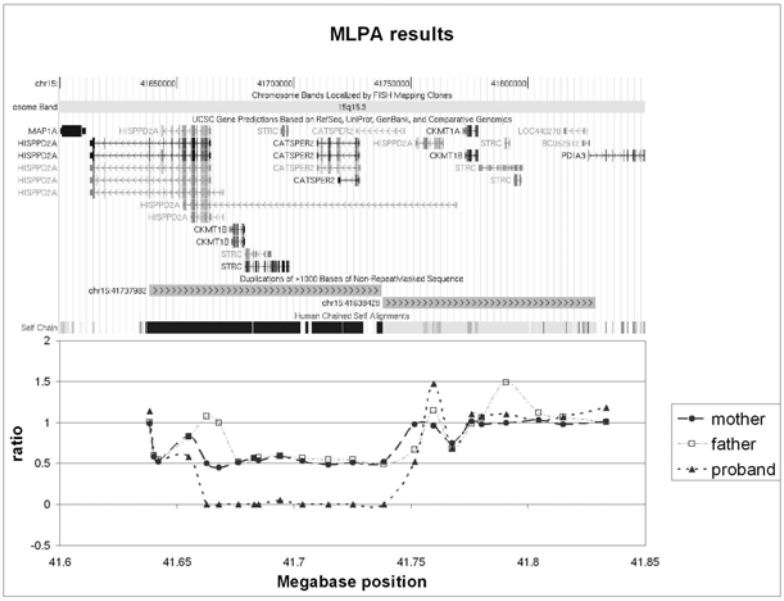

Figure 3. MLPA results on the proband and both parents. The upper panel shows the involved region using the UCSC browser, in which the involved genes and the two large duplicon blocks are displayed. The lower panel shows the ratio profiles of normalized MLPA data. Each data point represents a specific MLPA probe that discriminates between the proximal and distal repeat region. The homozygous deletion in the proband of the largest part of the proximal duplicon and the beginning of the distal duplicon is indicated by the closed triangles. The parents both show the heterozygous deletion in the region.

To confirm alterations involving this region reported in the DGV, 21 samples of the HapMap collection were tested with the developed MLPA set. Of these samples, 10 were reported with either a gain or a loss in the 15q15.3 region[1,10] and the remainder 11 were the closest family members, if they were available. Two additional samples with a duplication as reported in the DGV were unavailable for analysis. Six of the 10 available samples were described as having a deletion size of at least one duplicon. All six deletions were confirmed and they could be narrowed down to the contiguous deletion of the size of one duplicon.

In all six cases the deletion involved the largest part of the proximal duplicon, deleting or disrupting the active genes on that allele. The four other samples had the smaller alterations reported,[10] as discussed below. All MLPA data is available in the online supplemental table 2.

DISCUSSION

In this study we describe the detailed molecular characterization of a homozygous deletion of a seemingly normal variation locus at 15q15.3, inherited from non-consanguineous parents. Using array-CGH, a deletion at chromosome 15q15.3 containing a 100 kb tandem segmental duplication region was found. SNP-array analysis ruled out possible consanguinity of the parents and showed the deletion to be homozygous. The rearrangement deletes the proximal repeat sequence at chromosome 15q15.3 on both alleles. It is important to note that only three SNP were located in this area, so current standard diagnostic reporting algorithms will not detect the deletion. This might result in false negative calls for the involvement of this region in diagnostic settings.

Homozygous deletion of the 15q15.3 region has been reported in the DIS syndrome. However, mental retardation and/or structural congenital abnormalities were not reported in those cases. [16,17] The clinical overlap with our patient is thus the hearing loss and potentially the infertility. The more severe phenotype in our patient may represent one end of a broader phenotypic spectrum associated with homozygous deletion of 15q15.3, as was noted before in other syndromes.[26,27] Alternatively, the mental retardation and dysmorphic features are unrelated, or only partially related to his 15q15.3 homozygous deletion.

MLPA testing characterized the homozygous deletion in detail. Based on the assumption that the deletions are mediated by NAHR during meiosis a deletion of a full repeat-size is expected, which is about 90 kb (fig 3) and fits the MLPA results. The MLPA probe at 41.75 Mb shows a heterozygous deletion ratio for the patient and the father and a normal ratio for the mother. Consequently, the distal breakpoint of the father is located slightly more telomeric than in the mother and assuming NAHR as the responsible recombination mechanism, the proximal breakpoint in the father should also be more telomeric (fig 4).

The results also show that the father of the described proband has two copies of the region detected by two MLPA probes around 41.67 Mb, while the proband did not inherit any of these two copies (fig 3). These probes are located in a non-coding area. Likely the father has two copies of this region on the non-transmitted, intact allele. Similar copy number variation was also observed by the MLPA test performed on control samples and HapMap samples. Some more small CNVs in the most proximal part of the proximal duplicon is noted throughout the sample sets, involving MLPA probes in intronic regions in and around the *HISPPD2A* gene. These variations might represent smaller genomic changes similar to the four small alterations in the HapMap cases described by Perry and colleagues.[10] Currently, there are no cost effective methods to screen for structural variants smaller than ~3 kb in a genome wide approach.[11]

The relevance of the non-contiguous variations found in intronic regions with the developed MLPA set is thus unclear.
In order to estimate the frequency of this deletion we tested the genomic DNA of 45 persons with non-syndromic sensorineural hearing loss and 75 control persons. Neither heterozygous nor homozygous deletions were found. Interestingly, one sample in the patient group and two samples in the control group were found with a duplication of the proximal repeat part, with a size comparable to the observed deletion. This leads to the conclusion that having an extra copy of the four active genes does not present a phenotype and also supports the theory of NAHR as the main mechanism of recombination of this repeat region.

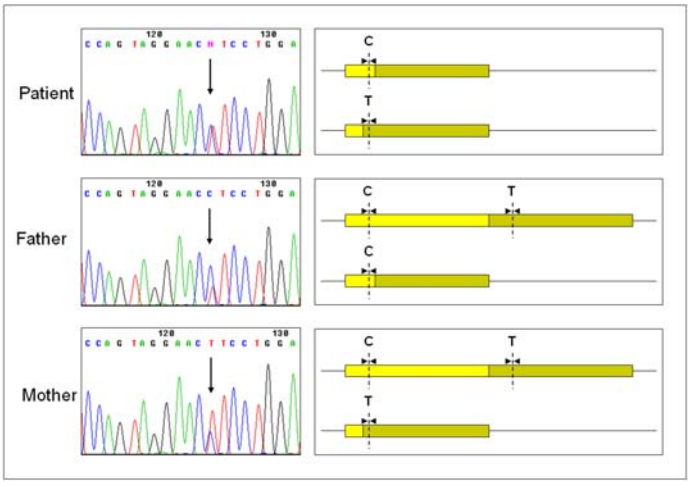

Figure 4. Sequence results of the paralogous sequence variant at base pair 41652116 of chromosome 15 in the proband and both parents. Sequence graphs show an equal ratio in the proband between the PSVs on both alleles, while the mother shows a 2:1 ratio for the C-allele and the father shows a 2:1 ratio for the T-allele (black arrows). The schematic drawing next to the sequence results show the inheritance of the different alleles that result in the ratio values of the PSV. The bright yellow bar is the proximal duplicon, the dark yellow bar is the distal duplicon and the arrowheads are the primers used to amplify the sequenced fragment. It also shows that in the maternal allele NAHR should have taken place proximal to the PSV, while in the paternal allele NAHR should have taken place distal to the PSV.

The investigated region on chromosome 15q15.3 was identified to be involved in normal variation (both gain and loss) in separate studies. [1,9,10,12] In the HapMap collection[19] that is analyzed in a part of these studies, 12 samples had a CNV reported at this location. Eight of these samples had a CNV of a continuous region greater than 30 kb in size, which indicates a full a deletion or duplication. This was confirmed with our MLPA test in the six available samples with the bigger alteration. Of these samples with alterations the size of a duplicon, 4 HapMap samples showed inheritance of the 15q15.3 CNV with either paternal or maternal transmission, while the remaining cases were individual samples. With regard to ethnic distribution, the 15q15.3 CNVs were present in the CEPH population, the Yoruban population from Nigeria and the Chinese and Japanese population. In our study we confirmed all these CNVs by using the targeted MLPA set. The existence of these deletions in different ethnic populations supports

that the presence of this variation is not restricted to one region or one founder family.
Pooling all in-house whole genome screened samples (n=300), locus specific data (n=109) and normal variation data published in the literature and summarized in the DGV database (n=320), a hemizygous deletion frequency of ~1.6% and a duplication frequency of ~0.7% (n=729) is found for this genomic region. This proves that the involvement of a normal variation locus with a hemizygous deletion frequency of 1.6% and a serious phenotype in the homozygous state is of importance for genetic testing. The presented data show that potentially important information may be missed when disregarding CNVs based on inheritance status and reported involvement in normal variation.
The fact that both pathogenic point mutations in the *STRC* gene[18] and homozygous deletions of the 15q53.3 region[16,17] have been reported to be associated with hearing loss, emphasizes the importance of considering the chromosome 15q15.3 locus in screening for the genetic cause of hearing loss.

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Patient consent: Informed consent was obtained of the patient and his parents described in this article.
Supplemental Data: Two tables are available online.

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