Branchio-Otic Syndrome Caused by a Genomic Rearrangement: Clinical Findings and Molecular Cytogenetic Studies in a Patient with a Pericentric Inversion of Chromosome 8

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Key Words
Branchio-otic syndrome · Branchio-oto-renal syndrome · \textit{EYA1} gene · Pericentric inversion of chromosome 8

Abstract
Branchio-oto-renal (BOR) syndrome is an autosomal dominantly inherited developmental disorder, which is characterized by anomalies of the ears, the branchial arches and the kidneys. It is caused by mutations in the genes \textit{EYA1}, \textit{SIX1} and \textit{SIX5}. Genomic rearrangements of chromosome 8 affecting the \textit{EYA1} gene have also been described. Owing to this fact, methods for the identification of abnormal copy numbers such as multiplex ligation-dependent probe amplification (MLPA) have been introduced as routine laboratory techniques for molecular diagnostics of BOR syndrome. The advantages of these techniques are clear compared to standard cytogenetic and array approaches as well as Southern blot. MLPA detects deletions or duplications of a part or the entire gene of interest, but not balanced structural aberrations such as inversions and translocations. Consequently, disruption of a gene by a genomic rearrangement may escape detection by a molecular genetic analysis, although this gene interruption results in haploinsufficiency and, therefore, causes the disease. In a patient with clinical features of BOR syndrome, such as hearing loss, preauricular fistulas and facial dysmorphisms, but no renal anomalies, neither sequencing of the 3 genes linked to BOR syndrome nor array comparative genomic hybridization and MLPA were able to uncover a causative mutation. By routine cytogenetic analysis, we finally identified a pericentric inversion of chromosome 8 in the affected female. High-resolution multicolor banding confirmed the chromosome 8 inversion and narrowed down the karyotype to 46,XX,inv(8)(p22q13). By applying fluorescence in situ hybridization, we narrowed down both breakpoints on chromosome 8 and found the \textit{EYA1} gene in q13.3 to be directly disrupted. We conclude that standard karyotyping should not be neglected in the genetic diagnostics of BOR syndrome or other Mendelian disorders, particularly when molecular testing failed to detect any causative alteration in patients with a convincing phenotype.
chial arch defects, preauricular pits/tags, and different re-
nal disorders [Melnick et al., 1975; Fraser et al., 1978]. Af-
ected individuals who lack renal dysplasia have been
described as having branchio-otic (BO) syndrome (MIM 602588). The disease has a high penetrance; however, the
clinical course of BOR/BO syndrome shows variable ex-
pressivity with a high inter- and intrafamilial variability
[Ou et al., 2008]. Most of the described cases result from
mutations in the EYA1 gene, which is located in 8q13.3 and
encodes a member of the drosophila eyes absent (EYA)
family of proteins. EYA1 was reported to act as a transcrip-
tional coactivator and as a phosphatase [Vervoort et al.,
2002; Krug et al., 2011]. Furthermore, EYA1 forms a com-
plex with SIX proteins, members of the Dach family (dachshund) and presumably additional not yet known
proteins [Ikeda et al., 2002; Li et al., 2003; Ahmed et al.,
2012]. The formation of this complex leads to a coactiva-
tion of SIX transcription factors which are essential for
normal development of several tissues and organs includ-
ing the second branchial arch, ears, eyes, and kidneys [Ike-
da et al., 2002; Li et al., 2003; Ahmed et al., 2012]. Up to
date, more than 110 different mutations in the EYA1 gene
(Molecular Otolaryngology Research Laboratory, Pendi-
dred/BOR homepage, http://www.healthcare.uiowa.edu/
labs/pendredandbor/) have been described to be associ-
ated with BOR/BO syndrome; they are randomly scattered
over the entire gene [Wang et al., 2012]. Few mutations
have also been found in genes encoding the SIX1 and SIX5
proteins [Krug et al., 2011]. The mutation detection rate in
patients with typical clinical features of BOR/BO syn-
drome has been reported with highest estimates of ~70%
[Krug et al., 2011]. This may be due to the genetic hetero-
geneity and clearly shows the difficulties in implementing
a strategy for molecular testing in patients with BOR/BO
syndrome. The spectrum of EYA1 mutations comprises
missense, nonsense, frameshift, and splice-site mutations
as well as small heterozygous deletions and complex ge-
nomic rearrangements. Therefore, the typical step-by-step
diagnostic approach of BOR/BO syndrome includes se-
quencing of EYA1, SIX1 and SIX5 as well as multiplex li-
gation-dependent probe amplification (MLPA) analysis
for the detection of possible deletions covering part or the
entire EYA1 gene. Nevertheless, genomic rearrangements
affecting EYA1 have been described to be a relatively fre-
cquent cause of BOR/BO syndrome and may be missed by
standard diagnostic tools [Kumar et al., 1992; Smith et al.,
1992; Vervoort et al., 2002]. Here, we report on a patient
showing major features of BO syndrome but missing renal
manifestations. Cytogenetic analysis revealed a pericentric
inversion of chromosome 8. By fluorescence in situ hy-
bridization (FISH), we demonstrate that one of the break-
points directly disrupts the EYA1 gene and thus represents
the genetic alteration causing BO syndrome in the affected
female.

Material and Methods

Case Report

The 43-year-old female patient presented with progressive sen-
soirneural hearing loss. Since the age of 3 years, she has worn hear-
ding devices. A computed tomography scan of the petrous bone
showed inner ear malformations combined with deformities of the
middle ear such as enlarged auditory tubes and absent mastoid
cells. Bilateral preauricular fistulae were treated by surgery at the
age of 21 years due to recurrent inflammation. She showed facial
dysmorphism with arched and sparse eyebrows, periorbital oede-
ma, sparse hair, a bulbous nose tip, a small upper lip, a receding
chin, a high arched palate, and no ear lobes (fig. 1). Renal ultra-
sound was normal. Her first pregnancy ended in a late-term abor-
tion (34th week), and it became apparent that the male fetus had
renal agenesis. Her 16-year-old daughter showed no clinical signs
of BO or BOR syndrome; renal ultrasound showed no abnormali-
ties, and preauricular fistulas were not found.

Laboratory Genetic Testing

After genetic counseling and collection of informed consents
of the patient and her parents, we obtained blood (heparin and
EDTA) samples of the trio. Genetic analyses were performed fol-
lowing the ethical guidelines of the institutes involved.

Molecular Genetic Testing

Genomic DNA was isolated from EDTA blood samples using
standard methods. Mutation analysis of the 16 coding exons of EYA1
(GenBank accession number NM_172058), the 2 coding exons of
SIX1 (GenBank accession number NM_005982), the 3 coding exons
of SIX5 (GenBank accession number NM_175875), and their flank-
ing intronic sequences was carried out by direct sequencing. Screen-
ing for deletions or duplications of the EYA1 gene was performed
using the MLPA kit SALSA P153-A2 according to the manufactur-
er’s instructions (MRC Holland, Amsterdam, The Netherlands).

Cytogenetic and Array Comparative Genomic Hybridization

Analysis

Routine cytogenetic investigations were performed on meta-
phase chromosomes derived from 72-h lymphocyte cultures ac-
cording to standard protocols. A minimum of 15 metaphases were
analyzed for each individual. Karyotypes were described according
to the International System for Human Cytogenetic Nomenclature
[Shaffer et al., 2009]. Array comparative genomic hybridization
(array CGH) was performed on genomic DNA of the patient using
the Human Genome 180K CGH Microarray (Agilent Technolo-
gies, Waldbronn, Germany) according to the recommendations of
the manufacturer. Interpretation was based on Human Genome
Build 36 (NCBI36/hg18).

High-Resolution Multicolor Banding

High-resolution multicolor banding (MCB) based on micro-
dissection-derived region-specific libraries of chromosome 8 was
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Fig. 1. Photographs of the patient affected by BOR syndrome. The diagnosis criteria established by Chang et al. [2004] are fulfilled. A, C The patient displays a long and narrow face and a receding chin. B Additionally, she has sparse hair which has also been associated with BOR syndrome. C She has worn hearing devices since she was a child; she also shows anomalies of the ear lobes. Preauricular tags were treated by surgery in her youth. Malformations of the middle and inner ear were confirmed by computed tomography (data not shown).

Fig. 2. Cytogenetic results of the normal and derivative chromosome 8. A The GTG banding pattern (550-band level) shows a pericentric inversion with one breakpoint in pter and the other breakpoint in q13 indicated by arrows. B MCB revealed an inverted banding pattern and fluorochrome profile in the region indicated by arrows and thus confirmed the inversion.

Results

Molecular Genetic Testing
Sequencing of the EYA1, SIX1 and SIX5 genes on DNA derived from the patient’s white blood cells showed no mutation. MLPA of the EYA1 gene revealed no copy number change.

Cytogenetic and Array Comparative Genomic Hybridization Analysis
Cytogenetic analysis showed a pericentric inversion on one of the chromosomes 8 in the index patient (fig. 2). The breakpoints were mapped close to 8pter and 8q13.
The parents of the index patient had a normal karyotype, with 46,XX and 46,XY. Array CGH analysis on genomic DNA of the index patient showed normal results: arr(1–22,X)×2, suggesting that the pericentric inversion of chromosome 8 was genetically balanced.

High-Resolution Multicolor Banding

After performing multicolor FISH analysis (fig. 2), the refined karyotype of the index patient was 46,XX,inv(8) (p22q13), describing a pericentric inversion of chromosome 8p22q13.

Mapping of the Breakpoints in 8p22 and 8q13.3 by FISH

By using various BAC and fosmid clones, we performed serial FISH analyses to delineate both breakpoints on chromosome 8. We first mapped the 8q13.3 breakpoint and identified the 2 BACs RP11-263H10 and RP11-242B23 and fosmid G248P8007F2, all covering part of EYA1 gene, as breakpoint-spanning clones: they gave a signal on wild type chromosome 8 as well as split signals on both arms of chromosome 8 with the pericentric inversion [inv(8)]. Chromosomes were counterstained with DAPI. Enlarged wild type chromosome 8 and inverted chromosome 8 with FISH signals of the breakpoint-spanning fosmid are shown on the bottom left and the top right, respectively.
breakpoint to 10–15 kb within intron 11 of the \textit{EYA1} gene (fig. 3).

We also mapped the 8p22 breakpoint and identified BAC RP11-1069K22 and fosmid G248P87904B12 which overlapped the breakpoint (online suppl. fig. 1). The breakpoint is located within the \textit{PCMI} gene (online suppl. fig. 1).

**Discussion**

In this study, we present clinical and genetic data of a female patient presenting with signs of BOR syndrome. According to improved clinical diagnostic criteria which have been introduced by Chang et al. [2004], there are 4 major criteria, i.e. branchial anomalies, deafness/hearing loss, preauricular pits, and renal anomalies, as well as several minor criteria, such as anomalies of the external, middle or inner ear, preauricular tags, facial asymmetry, and palate abnormalities [Chang et al., 2004]. Our patient displayed 2 major clinical features, namely sensorineural hearing loss and preauricular tags, and several minor features, e.g. anomalies of the outer, the middle and the inner ear as well as a long and narrow face. Altogether, she meets the diagnostic criteria of BO syndrome due to the lack of renal abnormalities. Additionally, our patient reported a stillborn child displaying renal agenesis in her first pregnancy which is in line with the observation that an extensive clinical variability exists, even between family members harboring the same mutation [Chang et al., 2004]. Unfortunately, we neither have detailed information on additional malformations nor the chromosomal status of the fetus. Thus, although we can only speculate on the genetic cause of the late-term miscarriage, we assume the presence of the same chromosome-8 inversion as seen in the mother.

Molecular genetic testing failed to detect a mutation in the genes \textit{EYA1} (8q13.3), \textit{SIX1} and \textit{SIX3}. Using highest clinical diagnostic standards, the detection rate of mutations in \textit{EYA1} was almost 70% in patients with a clear-cut phenotype [Chang et al., 2004; Krug et al., 2011]. Mutations in \textit{SIX5} and \textit{SIX1} are causative in 5% of the cases [Sanchez-Valle et al., 2010]. Several studies have demonstrated that in ~80% of mutation-positive cases, mutations could be detected by direct sequencing, whereas in ~20% of affected individuals, complex genomic rearrangements of chromosome 8, which likely affect expression of \textit{EYA1} on one allele, caused the phenotype [Chang et al., 2004; Sanchez-Valle et al., 2010]. The latter applies to the female patient reported here, as we identified a balanced pericentric chromosome-8 inversion with direct interruption of the \textit{EYA1} gene by 1 of the 2 breakpoints.

Various chromosomal rearrangements in patients with BOR syndrome have been reported so far, ranging from inversions via small insertions to large deletions. However, most of the reported rearrangements are unbalanced, and in some cases, other genes in addition to \textit{EYA1} were deleted leading to a contiguous gene syndrome in patients with BOR syndrome and additional clinical features [Chang et al., 2004]. Therefore, most of these rearrangements cannot be compared with our case. Vervoort et al. [2002] characterized and summarized genomic rearrangements detected by the means of sequencing, single-strand conformation polymorphism, Southern blot, and transcript analysis. They reported a single inversion in chromosome 8, which was associated with BOR syndrome [Vervoort et al., 2002]. This paracentric inversion was found in several members of a family with clinical signs of BOR syndrome. Breakpoint analysis was performed by Southern blot analysis and revealed one of the breakpoints in intron 1 and the second breakpoint outside the \textit{EYA1} gene. The study was published in 2002. Meanwhile, standard diagnostic methods for the detection of large genomic rearrangements have been improved by introducing tools to identify abnormal copy numbers, such as MLPA. However, MLPA does not allow a detection of balanced translocations and inversions, similar to array CGH. In our case, we narrowed down the inversion breakpoints on the p and q arm of chromosome 8 and found that the one in 8q13.3 disrupts the \textit{EYA1} gene and thus caused classical BO(R) syndrome in the patient. The second breakpoint in 8p22 is located within the \textit{PCMI} gene (online suppl. fig. 1). \textit{PCMI} encodes the centriolar material 1 protein, which is a component of centriolar satellites [Kubo et al., 1999]. \textit{PCMI}-containing centriolar satellites move along microtubules, i.e. toward centrosomes, and are involved in the microtubule- and dynactin-dependent recruitment of proteins to the centrosome [Kubo et al., 1999; Dammermann and Merdes, 2002]. Mutations in \textit{PCMI} have not been associated with any Mendelian disorder so far. However, loss of both the \textit{PCMI} gene and the encoded protein has been observed in breast and ovarian carcinomas [Armes et al., 2004; Pils et al., 2005; Venter et al., 2005] and papillary thyroid carcinoma [Corvi et al., 2000]. Thus, according to the current data, it seems unlikely that the phenotype of the patient with BO syndrome is caused or modified by direct interruption of \textit{PCMI}.  

DOI: 10.1159/000355436  

Cytogenet Genome Res 2014;142:1–6
Considering these results, we conclude that conventional cytogenetic approaches should be taken into account in the genetic diagnostics of patients showing clinical signs of BOR syndrome as well as other autosomal dominantly inherited conditions when molecular genetic analysis was negative.

Acknowledgements

We thank the patient and her family for their kind cooperation and their permission to publish genetic data and photographs. We declare that there are no conflicts of interest. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (KU 1240/6-1 to K.K.).

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