

ARTICLE

# Branchio–oto–renal syndrome: detection of *EYA1* and *SIX1* mutations in five out of six Danish families by combining linkage, MLPA and sequencing analyses

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The branchio–oto–renal (BOR) syndrome is an autosomal-dominant disorder characterized by hearing loss, branchial and renal anomalies. BOR is genetically heterogeneous and caused by mutations in *EYA1* (8q13.3), *SIX1* (14q23.1), *SIX5* (19q13.3) and in an unidentified gene on 1q31. We examined six Danish families with BOR syndrome by assessing linkage to BOR loci, by performing *EYA1* multiplex ligation-dependent probe amplification (MLPA) analysis for deletions and duplications and by sequencing of *EYA1*, *SIX1* and *SIX5*. We identified four *EYA1* mutations (c.920delG, IVS10–1G > A, IVS12 + 4A > G and p.Y591X) and one *SIX1* mutation (p.W122R), providing a molecular diagnosis in five out of the six families (83%). The present, yet preliminary, observation that renal and temporal bone malformations are less frequent in *SIX1*-related disease suggests a slightly different clinical profile compared to *EYA1*-related disease. Unidentified mutations impairing mRNA expression or further genetic heterogeneity may explain the lack of mutation finding in one family despite LOD score indications of *EYA1* involvement.

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**Keywords:** BOR syndrome; *EYA1*; *SIX1*; syndromic hearing impairment

## Introduction

The branchio–oto–renal (BOR) syndrome (OMIM 113650) is an autosomal-dominant developmental disorder, associated with hearing loss, branchial arch and renal anomalies.

The highly variable phenotype in patients with BOR syndrome comprises hearing loss (93%), preauricular pits (82%), renal anomalies (67%), branchial fistulae (49%), deformed pinnae (36%) and external auditory canal stenosis (29%).<sup>2</sup> Less frequent findings include preauricular tags (13%) and lacrimal duct aplasia (11%).<sup>2</sup> A set of diagnostic criteria was suggested by Chang *et al.*<sup>3</sup> Based on clinical presentation, the prevalence of BOR was estimated to be 1:40 000, and to affect approximately 2% of profoundly deaf children.<sup>4</sup>

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Hearing loss is a crucial finding, presenting as sensorineural, conductive or mixed type with about equal distribution of the three types of hearing loss. The hearing impairment may be progressive and fluctuating.<sup>5</sup> Temporal bone abnormalities include enlarged vestibular aqueduct (EVA), hypoplastic cochleae and hypoplastic semicircular canals,<sup>6–9</sup> but it should be mentioned that molecular diagnosis was absent in these early reports. In one study, applying a standardized set of measurements, 76.9% of patients had either unilateral or bilateral CT abnormalities.<sup>7</sup> A recent study reports EVA in a patient with a *SIX1* mutation.<sup>10</sup>

BOR is genetically heterogeneous and is associated with mutations in *EYA1* on chromosome 8q13.3, *SIX1* on 14q23.1, and a third BOR locus, *BOR2*, on 1q31.<sup>11–13</sup> After submission of this article, the *SIX1* homologue *SIX5* (19q13.3) has been found to be mutated in BOR patients.<sup>14</sup> At least 116 different *EYA1* mutations and rearrangements have been reported (isoform B).<sup>3,15,16</sup> By contrast, only few mutations have been reported in the *SIX* genes. Thus, so far, five unrelated families have been published with three different mutations in *SIX1*,<sup>10,17</sup> and five BOR individuals have been identified with four different *SIX5* missense mutations.<sup>14</sup>

Alternative splicing of *EYA1* produces four transcript variants, expressing three different isoforms with the longest isoform having 18 exons.<sup>3</sup> Eya family members (Eya1–4) are defined by a conserved ~275 amino-acid C-terminal domain, referred to as the Eya domain and which possesses protein phosphatase activity.<sup>18</sup> Eya proteins, including Eya1, function as co-activators in transcriptional regulation by Dach proteins and Six proteins (including Six1 and Six5) during mammalian organogenesis.<sup>17–19</sup> Eya has no apparent DNA-binding ability and is translocated from the cytoplasm to the nucleus by Six and serves as a co-activator of Six in the regulation of downstream genes.<sup>19,20</sup> Both Eya and Dach may be required for Six-mediated gene activation.<sup>19</sup> The recently discovered protein phosphatase function of the Eya family is thought to switch Six-Dach from a repressor to an activator of transcription via dephosphorylation.<sup>18,19</sup>

The aim of this study was to identify the disease causing mutations in six Danish families diagnosed with BOR, of which five have been published with clinical description previously. Family information was updated, and linkage analysis to *EYA1*, *SIX1* and *BOR2* locus, respectively, was performed, followed by *EYA1* multiplex ligation-dependent probe amplification (MLPA) analysis, and sequencing of *EYA1*, *SIX1* and *SIX5*.

## Materials and methods

### Patients and families

We used the clinical criteria suggested by Chang *et al*<sup>3</sup> in which an individual must fulfil one of the following

criteria to be classified as affected with BOR: (A) fulfil at least three major criteria (branchial anomalies, deafness, pre-auricular pits, renal anomalies), or (B) fulfil two major criteria and at least two minor criteria (external, middle or inner ear anomalies, pre-auricular tags, and possibly also have other features such as facial asymmetry or palate abnormalities) or (C) fulfil one major criteria and have an affected first-degree relative meeting the criteria for BOR.<sup>3</sup> Six Danish families diagnosed with BOR participated in the study (Figure 1). Family 1,<sup>21</sup> 3,<sup>22</sup> 4,<sup>22</sup> 5<sup>6</sup> and 6<sup>22</sup> have been described previously, whereas family 2 is new. The clinical data were compiled from previous publications and from available medical records from different hospitals. Venous blood was drawn from all available patients and relatives. The study was approved by the Danish Research Ethical Committee (reference numbers KF 01–234/02 and KF 01–108/03).

### DNA isolation and chromosome analysis

DNA was extracted from peripheral blood samples using standard methods. Chromosome analysis of cultivated leucocytes in a patient from family 4 (individual III:5) followed standard procedures for karyotyping.

### Marker analysis

The following markers were chosen to cover the three BOR loci: D8S381, D8S1795 and D8S279 for linkage to *EYA1*; D1S2757 for linkage to the *BOR2* locus; and D14S980 and D14S290 for linkage to *SIX1*. In one family, additional markers were used to saturate the *BOR2* region: D1S2640, D1S238, D1S2757, D1S1660, D1S2654, D1S2872 and D1S249. The forward primers for the markers were labelled using  $\gamma$ -[<sup>33</sup>P]-dATP (Hartmann Analytic, Helsingborg, Sweden) and T4-DNA-polynucleotide kinase (Fermentas, Braunschweig, Germany) according to the manufacturer's protocol. PCR was carried out using 50 ng DNA and Taq-polymerase (New England Biolabs, USA), and PCR products were separated by 5% acrylamide gel electrophoresis and exposed to X-ray films.

### Linkage analysis

The two-point LOD scores and multipoint LOD scores were calculated using the computer program MLINK and LINKMAP routines of the FASTLINK software version 2.2. The multipoint LOD score was calculated based on microsatellite markers as reported in the GDB database (www.gdb.org). The LOD scores were calculated by using a dominant model with complete penetrance (100%).

### MLPA analysis

MLPA analysis detects deletions or duplications of one or more exons, but not inversions. The MLPA-EYA1 kit (P153) was designed by MRC-Holland, Amsterdam, The Netherlands, upon the authors' suggestion. The *EYA1* probe mix contains 17 probes for *EYA1* exons and 14 control probes.

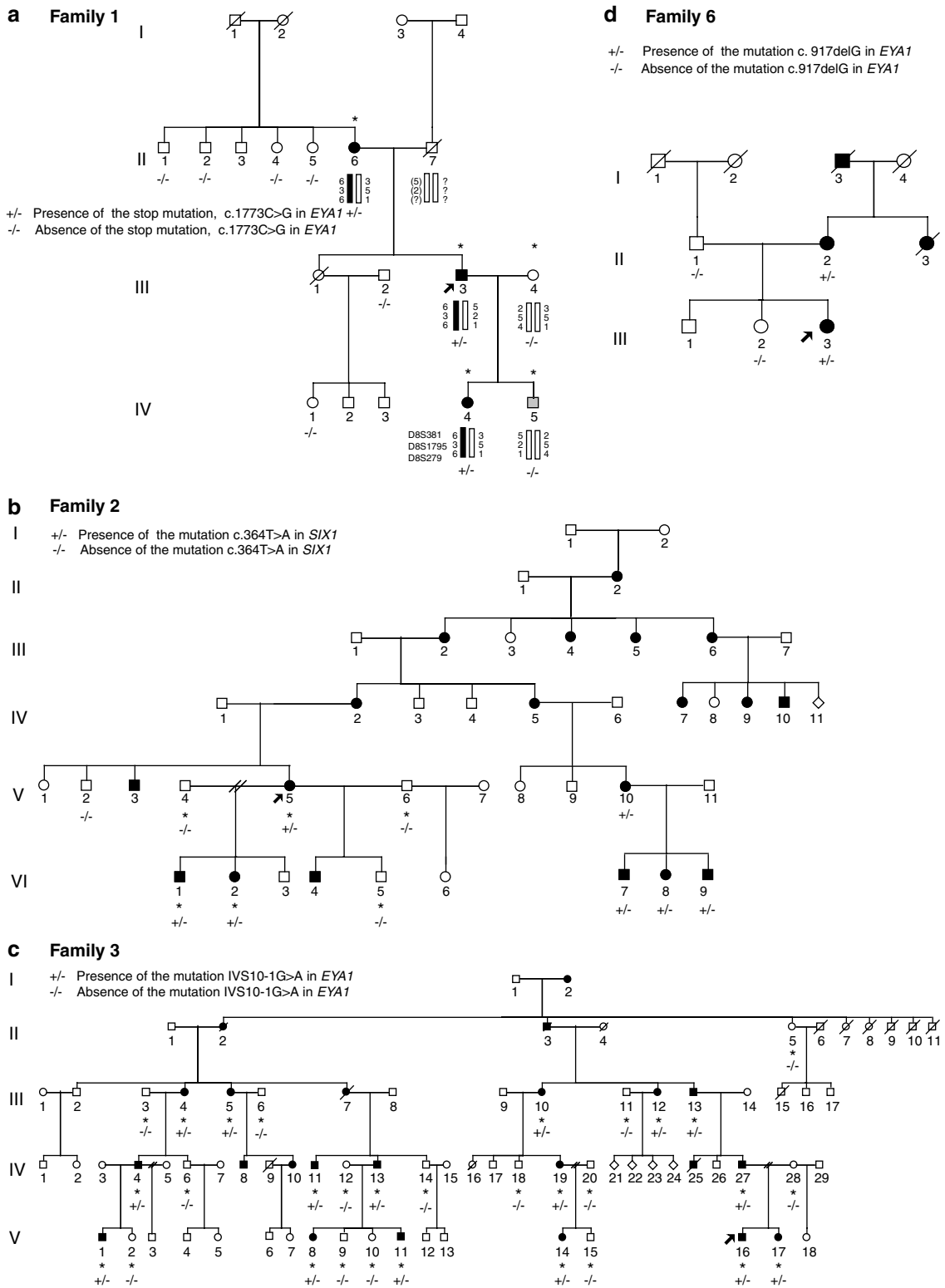
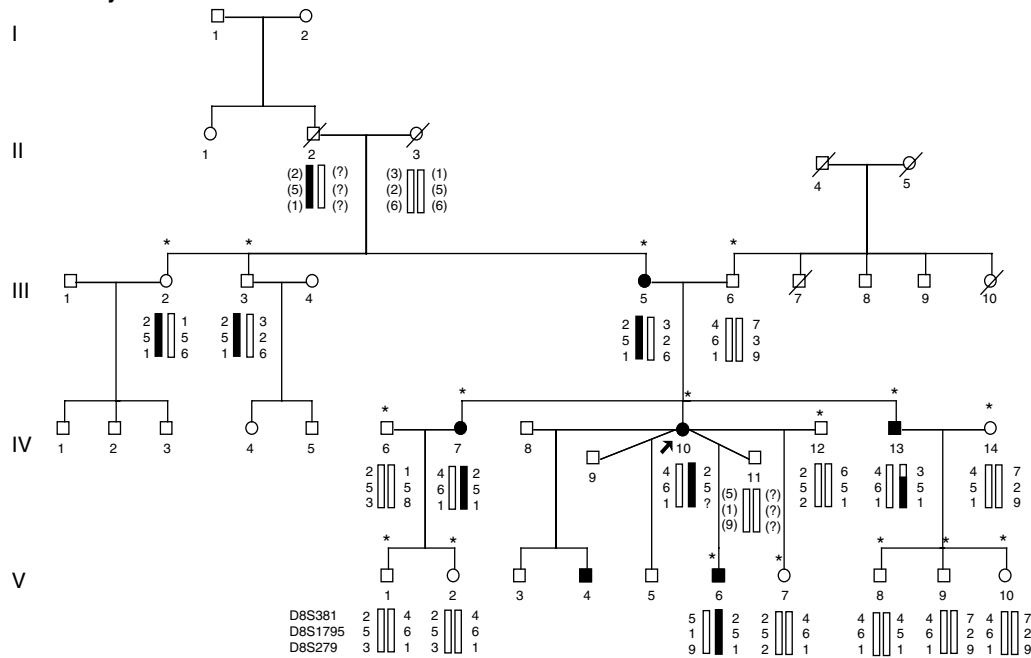
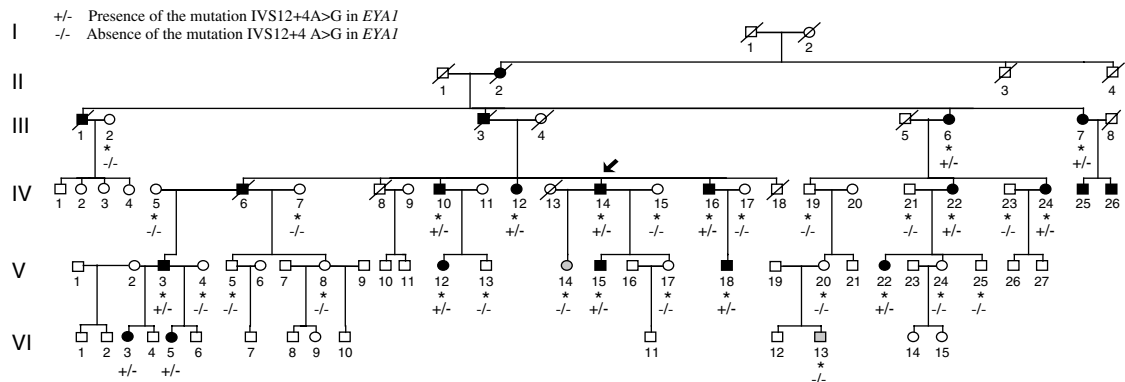


Figure 1 Continued.

**e Family 4****f Family 5**

**Figure 1** Pedigrees of the Danish BOR families 1–6. Proband is indicated by arrows. Asterisks (\*) indicate individuals who were included in linkage analysis. (a) Pedigree of family 1 with haplotypes for *EYA1* on chromosome 8 with markers D8S381, D8S1795 and D8S279. The c.1773C>G mutation segregated with BOR in the family. Individual IV-5 turned out to be a phenocopy. (b) Pedigree showing family 2. The c.364T>A mutation in *SIX1* segregated with BOR in the family. Individuals V-10, VI:7, VI:8 and VI:9 were not included in linkage analysis due to late arrival of the blood samples. (c) Pedigree showing family 3. The mutation, ISV10–1G>A (c.1141–1G>A), in *EYA1* segregated with BOR in the family. (d) Pedigree showing family 6. The c.917delG mutation in *EYA1* segregated with BOR in the family. (e) Pedigree showing family 4 with haplotypes for *EYA1* on chromosome 8 with marker D8S381, D8S1795 and D8S279. No mutation was identified in this family. (f) Pedigree showing family 5. The mutation, ISV12+4A>G (c.1360A>G), in *EYA1* segregated with BOR in the family. Individuals V:14 and VI:13 are phenocopies.

To test the MLPA-*EYA1* kit, a DNA sample carrying an *EYA1* deletion previously identified by other methods was reanalysed using the kit and the kit reliably detected the deletion (Supplementary Figure 1). In the present study, we planned – prior to sequencing (17 PCRs per patient) of *EYA1* – to perform MLPA analysis (one ligation and one PCR per patient), since deletions and duplications in *EYA1* have been identified in up to 20% of *EYA1* patients,<sup>3</sup> and

when applied in this order, the two methods provide the most time- and cost-efficient strategy for the molecular diagnosis of *EYA1*.

**PCR and mutation analysis in *EYA1* and *SIX1***

Primers were designed to amplify exons and surrounding intronic regions of *EYA1* (GenBank nos. AJ000098 and NM\_172060.1 (for exon 1)), *SIX1* (GenBank no.

NM\_005982) and *SIX5* (NM\_175875.3), respectively (Supplementary Table 1). PCR-amplified samples were sequenced using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All 17 coding *EYA1* exons were sequenced in family 1–6 and in addition *EYA1* non-coding exons in family 4. Both coding exons of *SIX1* in family 2 were sequenced. In order to study the segregation of the mutations with the disease in each family, we used either restriction enzyme digesting or sequencing. All detected mutations (Table 1) and polymorphisms (Supplementary Table 2) were investigated in at least 50 Danish control individuals, if the sequence variation was not reported before in the NCBI ENTREZ SNP database.

### Prediction of consequences of splice site mutations

The effect of the identified splice site mutations on mRNA splicing was predicted by using the prediction server: Automated Splice Site Analysis (<https://splice.cmh.edu/>), which correlates the difference in information content ( $R_i$ ) between the normal and the variant splice site sequence.<sup>23,24</sup> The minimal quantity of information required for splicing has been determined to be 2.4 bits.<sup>23</sup>

### RT-PCR analysis

In order to investigate the effect of the splice site mutations IVS10–1G>A and IVS12+4A>G, respectively, we studied the expression of *EYA1* in blood and skin. RT-PCR with primers amplifying exons 10–12 (forward: 5'-ccactt cagttcccttga-3'; reverse: 5'-ttaccctgtctgtagcggaag-3') and exons 8–13 (forward: 5'-gtcagagttcagatgggaaa-3'; reverse: 5'-agtttgctccggagtgatg-3'), respectively, was performed on cDNA from blood and cultured fibroblasts. As control, PCR with internal primers (forward: 5'-ataactttggaacagatggc-3'; reverse: 5'-ctctttaccctgtctgtagc-3') in exon 12 was performed. The PCR products were subsequently sequenced to verify the sequence amplified by the primers.

### Results Families

The identified mutations and the clinical data in the six BOR families are summarized in Table 1 and considerably more in detail in Supplementary Table 3. Below, the individual families are presented.

**Family 1** The symptoms of the affected individuals in family 1 have previously been described.<sup>21</sup> Briefly, the

**Table 1** Summary of clinical data and identified mutations in *EYA1* and *SIX1* in six Danish BOR families

	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6
Nucleotide change <sup>a</sup>	c.1773C>G	c.364T>A	c.1141-1G>A	Not detected	c.1360+4A>G	c.920delG
Gene	<i>EYA1</i>	<i>SIX1</i>	<i>EYA1</i>		<i>EYA1</i>	<i>EYA1</i>
Amino acid change	p.Y591X	p.W122R	p.E380fsX387 <sup>b</sup>		p.G454fsX461 <sup>b</sup>	p.R307fsX365
<i>EYA1</i> -MLPA analysis	Normal	Normal	Normal	Normal	Normal	Normal
No. of patients <sup>c</sup>	3 (3)	19 (7)	23 (16)	6	21 (15)	4 (2)
Female:Male	2F:1M	13F:6M	12F:11M	3F:3M	10F:11M	3F:1M
<i>Clinical findings<sup>d</sup></i>						
<i>Major criteria</i>						
Hearing impairment	3/3	14/15	17/18	6/6	21/21	3/3
Branchial defects	3/3	1/11	12/14	3/4	19/21	3/3
Preauricular defects	3/3	5/11	9/9	2/2	18/21	3/3
Renal anomalies	0/3	0/3	3/7	1/1	4/7	2/2
<i>Minor criteria</i>						
Malformed auricles	3/3	—	4/7	—	16/17	1/2
Middle ear defects	1/1	0/3	1/1	1/1	6/6	2/2
Inner ear defects	0/1	2/3	2/3	—	4/5	1/1
Facial nerve paresis	2/3	NI	3/3	—	1/1	—
<i>Other</i>						
Mental retardation	—	5/11	1/18	—	—	—
Preterm delivery	—	—	—	—	—	1/4

In family 6, one preterm born child could not be assessed in terms of hearing impairment and minor physical abnormalities. Individuals (in families 1 and 5) who were judged to be phenocopies have not been included in the table.

<sup>a</sup>Nucleotide numbering refers to the *EYA1* and *SIX1* cDNA sequence no. AJ000098 and NM\_005982, respectively, in GenBank. All mutations were present in the heterozygous state.

<sup>b</sup>The mutation is thought to affect mRNA splicing.

<sup>c</sup>The total number of patients is given, followed by the number of patients with a verified BOR mutation, in parenthesis.

<sup>d</sup>The total number of patients that have been diagnosed clinically with the particular anomaly is given before the slash. After the slash is given the number of examined patients for a particular anomaly. Note, that clinical information was available for some patients, from whom we did not have DNA for molecular diagnosis. We have listed all known patients in each family, including deceased affected despite non-availability of samples to verify their state as mutation carriers.

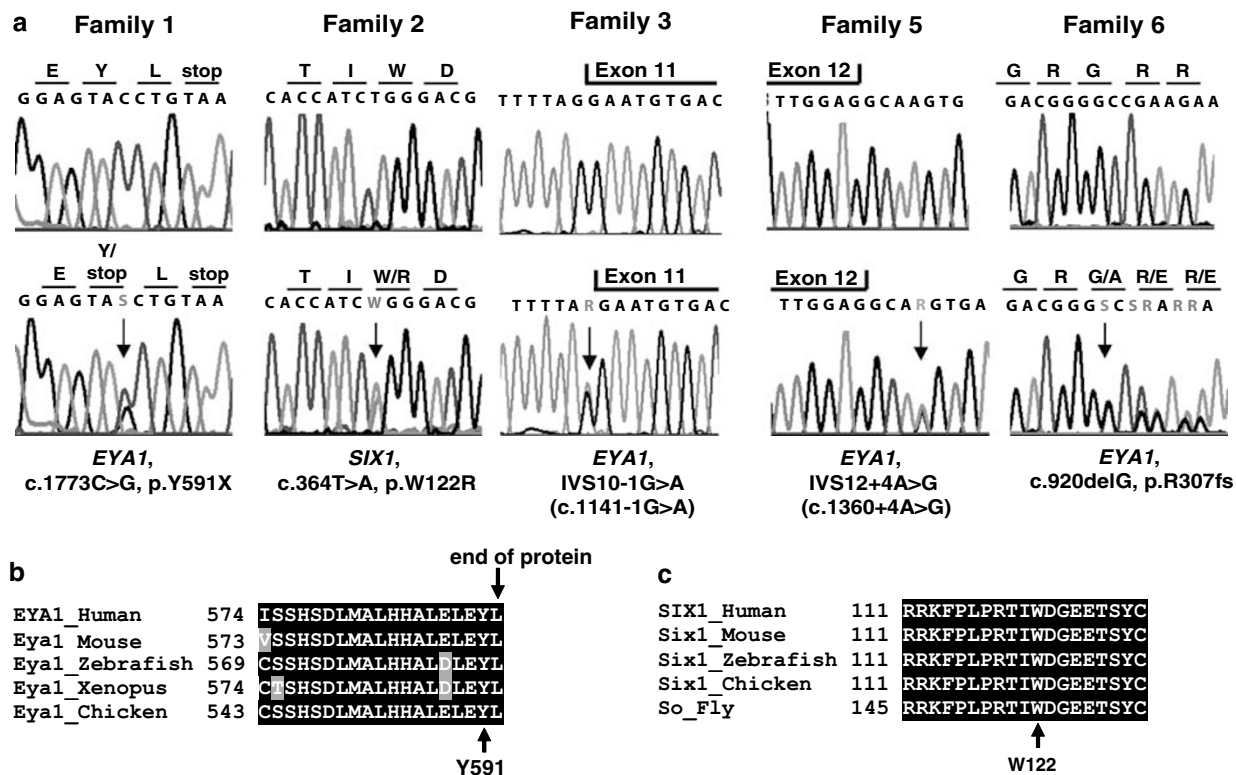
three affected individuals (II:6, III:3 and IV:4; Figure 1a) have hearing loss, branchial anomalies, preauricular pits and external ear deformity (representative audiograms are shown in Supplementary Figure 2). One individual (IV:1) who was otherwise healthy had external ear deformity. Moreover, one individual (IV:5) had congenital profound hearing impairment, using sign language (ie a very different hearing phenotype than the other affected individuals, see Supplementary Table 3), and no preauricular or branchial abnormalities and was therefore considered a phenocopy and unaffected with respect to BOR. Sequencing of *GJB2* in this individual did not identify any mutations nor were the mutations *del(GJB6-D13S1830)* and *del(GJB6-D13S1854)* present in the patient. Renal ultrasound was performed as a follow-up in three individuals (III:3, IV:4 and IV:5), and turned out normal.

Family 1 is too small to obtain a statistically significant LOD score. Five members were included in linkage analysis for *EYA1* and *SIX1*, and 12 individuals were included in linkage analysis to the *BOR2* locus. The disease mutation seemed to be *de novo* in generation II, since individuals in generation I as well as the five siblings of the oldest patient (II:6) were reported to be healthy (Figure 1a). Furthermore, siblings of II:6 shared the disease haplotype with individual II:6 (data not shown), which supported the notion of a *de*

*novo* mutation in II:6. The two-point LOD score did not reach statistical significance in favour of any of the loci.

MLPA analysis of *EYA1* was normal, but sequencing of *EYA1* revealed a novel mutation, c.1773C>G, which results in the substitution of a tyrosine codon for a stop codon at position 591 (p.Y591X), two amino acids before the wild-type stop codon in *EYA1* (Figure 2a). A restriction enzyme digest analysis confirmed the presence of the mutation in all three affected individuals (II:6, III:3 and IV:4), and the absence of the mutation in IV:5 and the other clinically unaffected individuals in the family (Figure 1a). The mutation was absent in 356 control chromosomes. Furthermore, the deleted Y591 and L592 are conserved in *EYA1* from species such as mouse, chicken, frog and zebrafish (Figure 2b), and in the human *EYA1* homologues *EYA2* and *EYA4*. *EYA3* has phenylalanine at position 591, which is nearly equivalent to a tyrosine residue. The evolutionary conservation of p.Y591 and p.L592, the co-segregation of the c.1773C>G mutation with the disease and the absence of the mutation in control individuals strongly indicate that this mutation underlies BOR in family 1.

**Family 2** Family 2 is reported for the first time as part of this study, and DNA was collected from eight individuals,



**Figure 2** (a) Sequence data for each of the mutations compared to a normal control. Each mutation is heterozygous in affected individuals. (b) Evolutionary conservation of *EYA1* amino acids Y591 and L592. (c) Evolutionary conservation of *SIX1* amino acid W122.

**Table 2** Two-point LOD score result in family 4

Gene	Marker	LOD score at $\theta =$						
		0.0	0.01	0.05	0.10	0.20	0.30	0.40
EYA1	D8S381	$-\infty$	0.27	0.91	1.10	1.11	0.89	0.52
	D8S1795	2.32	2.30	2.20	2.04	1.64	1.15	0.59
	D8S279	0.24	0.25	0.27	0.27	0.22	0.13	0.04
	D8S1795 <sup>a</sup>	2.71	2.67	2.49	2.25	1.75	1.19	0.60
BOR2	D1S2757	$-\infty$	-6.69	-3.30	-1.94	-0.75	-0.23	-0.01
SIX1	D14S980	$-\infty$	-7.31	-3.91	-2.52	-1.26	-0.65	-0.28
	D14S290	$-\infty$	-9.02	-4.93	-3.26	-1.73	-0.93	-0.40

<sup>a</sup>Two-point LOD score was calculated excluding individual III:2, III:3, II:2 and II:3, because of a strong suspicion of a *de novo* mutation in III:5.

four of whom were affected (V:5, VI:1, VI:2 and V:10; Figure 1b). Individual V:5 has progressive sensorineural hearing loss and unilateral preauricular pit and fistula. Two of her three children (VI:1 and VI:2) are affected and both have sensorineural hearing loss, but neither branchial nor preauricular anomalies. Representative audiograms are shown in Supplementary Figure 3. There were no subjective complaints indicating renal disease. In addition, psychiatric illness and mental retardation were reported in some family members (Supplementary Table 3). Inspection of the haplotypes did not exclude linkage to any of the three BOR loci. Neither MLPA analysis nor sequencing of *EYA1* did reveal any abnormalities. By contrast, sequencing of *SIX1* identified the sequence change c.364T>A in exon 1, leading to a substitution of tryptophan for arginine in position 122 of *SIX1* (p.W122R) (Figure 2a). The mutation segregated with the disease in the family, and the mutation was absent in 140 control chromosomes as determined by sequencing. W122 is located in the Six domain of human *SIX1* protein<sup>17</sup> and is conserved in Six1 from species such as mouse, chicken and zebrafish and in the fly orthologue So (Figure 2c), as well as in all six human *SIX* homologues. The evolutionary conservation of W122, the co-segregation of the c.364T>A mutation with the disease and the absence of the mutation in control individuals strongly indicate that this mutation underlies BOR in family 2.

**Family 3** Family 3 was initially reported in 1986 (family 3 in Gimsing and Dyrmosen<sup>22</sup>) with classical BOR in five generations and affecting all organ systems (Table 1). Sixteen patients and seven unaffected at risk individuals as well as relevant spouses were included in linkage analysis. We obtained a significant two-point LOD score showing linkage to *EYA1* and could exclude linkage to the *BOR2* locus (meaning that the marker was not informative, but haplotype inspection excluded this locus) and to *SIX1*. MLPA analysis for *EYA1* was negative, but sequencing of *EYA1* revealed a splice site mutation in intron 10, IVS10-1G>A (1141-1G>A) (Figure 2a). The mutation

involves the first nucleotide of the splice acceptor site in the intron and is therefore expected to result in aberrant splicing. Restriction-enzyme analysis showed that the mutation segregated with BOR in the family (Figure 1c). In one individual (V:11), the demonstration of the mutation confirmed a strong suspicion of BOR, since this 6-year-old boy had bilateral preauricular fistulae, but no detectable hearing impairment, so far. Renal ultrasound of the kidneys was unremarkable (Supplementary Table 3). The IVS10-1G>A mutation was not found in 100 control chromosomes. The nucleotide is conserved between species (<http://genome.ucsc.edu/>), suggesting a functional importance. Notably, the same nucleotide change has previously been reported in a BOR patient (patient MORL 2410),<sup>3</sup> strongly implying that it is pathogenic.

In order to predict the effect of the IVS10-1G>A mutation on mRNA splicing, we used the prediction-server for splice mutations to calculate the  $R_i$  values for normal and variant nucleotide. The  $R_i$  value for the normal splice site in exon 11 declines from 6.3 to -16.1 bits, whereas the  $R_i$  for an activated new/cryptic splice site, one nucleotide upstream from the natural splice site, increases from -11.6 to 3.2 bits, which suggests that the new cryptic splice site is active, and the natural splice site becomes inactivated. Thus, the IVS10-1G>A mutation was predicted to move the splice site one nucleotide and introduce a frameshift at codon 380. Provided this mRNA is stable, it is expected to cause a protein with a disrupted phosphatase domain. To further examine the functional effect of this splice site mutation, we first established that *EYA1* is expressed in fibroblasts, but not in blood, which agrees with previous findings.<sup>25,26</sup> We thereafter obtained a skin biopsy from an affected individual (IV:13) to obtain fibroblast cDNA from a person with the mutation. However, no variant band was detected by agarose gel electrophoresis and sequencing of RT-PCR products only detected mRNA with normal splicing. An explanation could be that the mRNA expressed from the 'abnormal' allele is unstable, whereby only the normal allele would be amplified by PCR.

**Family 4** Family 4, briefly described in 1986 (family 1 in Gimsing and Dyrmosen<sup>22</sup>), was updated as part of this study. There were no renal complaints, but otherwise typical features of BOR (Table 1). The family history indicated a *de novo* mutation in individual III:5 (Figure 1e). Her karyotype showed normal female chromosome constitution, 46, XX. Seventeen family members (five patients, eight at-risk individuals and four spouses) were included in the linkage analysis, and the two-point LOD scores indicated linkage to *EYA1* for marker D8S1795 ( $LOD_{D8S1795} = 2.32$ ), and seemed to exclude linkage to both the *BOR2* locus and *SIX1* (Table 2). The haplotype inspection raised strong suspicion of the possibility of a *de novo* *EYA1* mutation in our proband's mother (Figure 1e; III:5). A re-calculation

assuming such an event, excluding III:2, III:3, II:2 and II:3 gave a higher LOD score of 2.71 with marker D8S1795 at  $\theta=0.0$ , which may be considered a significant LOD score once the disease locus is known.<sup>27</sup> Sequencing of coding and non-coding exons in *EYAI* only revealed polymorphisms (Supplementary Table 2) but no mutations. A new polymorphism (IVS9+107A>G) was introduced by a spouse and did not segregate with the disease. Furthermore, IVS9+107A>G was found in 16/56 normal controls, thereby being a frequent polymorphism in the Danish population. Forty-five base pairs in the 5'UTR of *EYAI* (5'-AAACCAATAAGGTTAGGACAAGAGAATAGCTGTGGTTTGCCTTGC-3') could not be sequenced. This stretch of DNA is located between two known polymorphisms (delT (rs35162278) and delAA (rs10635780, rs34976941)) found in the family. *EYAI* MLPA analysis was normal, and a CGH study did not detect any submicroscopic imbalances (data not shown). Furthermore, *SIX5* was sequenced in this family, but no disease mutation was identified. By these analyses we have so far been unable to identify the molecular cause of BOR in this family.

**Family 5** Family 5 is a five-generation, large family with 13 patients and eight at-risk individuals available for linkage analysis. A previously description focussed on the occurrence of temporal bone abnormalities in 1991.<sup>6</sup> All three BOR patients with *EYAI* mutations (Figure 1f; individuals IV:10, IV:16 and V:12), who had a CT scan of the temporal bone reported in Ostri *et al.*,<sup>6</sup> shared the following abnormalities: hypoplastic middle ear cavity, severely malformed ossicular chain and hypoplastic cochleae, with only two coils and no evidence of enlarged endolymphatic duct.<sup>6</sup> As part of the study we have extended the analysis of the family by including the youngest generation VI (Figure 1f). This family had end-stage renal disease, and otherwise typical BOR findings (Table 1). The linkage analysis with chromosome 8 markers showed linkage to *EYAI* by two-point LOD score and multipoint LOD score and excluded linkage to both the BOR2 locus and *SIX1*. *EYAI*-MLPA analysis was normal, but sequencing of *EYAI* revealed a splice mutation, IVS12+4A>G (c.1360+4A>G) (Figure 2a). Sequencing of DNA from the family members showed co-segregation of the mutation with the disease (Figure 1f). Sequencing of 226 control chromosomes for this splice site mutation did not detect the mutation. The nucleotide of the splice mutation is conserved in species such as mouse, dog, chicken and opossum (<http://genome.ucsc.edu/>), suggesting a functional importance. In contrast to the wild-type allele, the mutated allele was not detected by standard RT-PCR on cultivated fibroblasts from an affected individual (IV:14). By nested RT-PCR, however, we detected abnormally spliced *EYAI* mRNA in the patient, in which exons 11 and 12 were deleted and a fragment of intron 10 was inserted instead. Taken together, these results indicate that

the c.1360+4A>G splice site mutation results in abnormally spliced mRNA that is very unstable.

**Family 6** Family 6 is a small three-generation family, published in 1986 (family 2 in Gimsing and Dyrmosé<sup>22</sup>). Individual I:3 (maternal grandfather of our proband, III:3 in Figure 1d) had profound hearing loss, bilateral branchial sinuses and a unilateral preauricular pit. He had a unilateral hypoplastic kidney, cystic parenchyme and renal failure treated with dialysis from age 55. Furthermore, individual I:3 had a malformed cochlea and malformed ossicles and hypoplastic cavity of the middle ear. His daughter (II:2) had stationary sensorineural hearing loss, bilateral branchial sinuses, unilateral preauricular tag, bilaterally malformed ossicles and normal kidneys as determined from by intravenous urography. Her sister (II:3) died 3 h after delivery and had bilateral agenesis of the kidneys (Potter's syndrome). Individual II:2 has three children of whom one is affected (III:3). Individual III:3 had conductive hearing loss, unilateral branchial fistula, unilateral preauricular pit but no renal pathology as determined from a normal intravenous urography at age 7,<sup>22</sup> and no subjective complaints at age 27 (Table 1). DNA was available from only four individuals and linkage analysis was therefore not feasible. *EYAI* MLPA analysis was normal. By contrast, sequencing of *EYAI* revealed a deletion, c.920delG, in exon 8 in *EYAI* (Figure 2a). The deletion leads to a frameshift at codon 307 just before the phosphatase domain and a stop codon at amino acid 365. Restriction enzyme and sequencing analysis confirmed the co-segregation of the mutation with the disease in the family. The mutation was not present in 100 control chromosomes.

## Discussion

In our study of six Danish BOR families, we identified the disease causing mutation in five families: four *EYAI* mutations (c. 1773C>G, IVS10-1G>A, IVS12+4A>G and c.920delG) and one *SIX1* mutation (c.364T>A).

The experience regarding *SIX1* is very limited with only four different *SIX1* mutations (in six unrelated families) known so far: Y129C,<sup>10,17,28</sup> delE133,<sup>17,29</sup> R110W<sup>17</sup> and now W122R, identified in our study. The W122R mutation, similar to the R110W and W122R mutation, is located in the SIX domain of the SIX1 protein, which is critical for the interaction with *EYAI*.<sup>17</sup> The Y129C and the delE133 mutations are located in the homeodomain of the SIX1 protein, which is critical for DNA binding.<sup>17</sup> Ruf *et al*<sup>17</sup> showed the R110W mutation to reduce the affinity of the *EYAI*-*SIX1* interaction, but not to affect the *SIX1*-DNA interaction, whereas the two homeobox mutations (Y129C and delE133) seemed to reduce both the *SIX1*-DNA and *EYAI*-*SIX1* interactions. The W122R mutation in the SIX



domain in our study is therefore expected to reduce the affinity for EYA1, but not for DNA.

Ruf *et al*<sup>17,28</sup> identified the Y129C missense mutation in the Anglo Saxon-Australian family underlying the report of the *BOS3* locus, which has been used to describe BOR phenotype, but without renal anomalies (two patients in this family developed renal carcinomas, which was most likely coincidental). The same mutation was found in a large Japanese family with autosomal-dominant inheritance of mixed progressive hearing loss, unilateral EVA and unilateral ear pit, but no branchial and renal anomalies.<sup>10</sup> Unfortunately, the authors only got permission to perform clinical and molecular studies of the proband.<sup>10</sup> The missense mutation, R110W, was found in two unrelated German families, who had hearing loss, preauricular and branchial but no renal anomalies.<sup>17</sup> Finally, the delE133 mutation was found in the same Swiss family, used for the identification of the non-syndromic hearing impairment, DFNA23 locus, who after re-investigation surprisingly turned out to have discrete renal anomalies.<sup>17,29</sup> In our family 2 with the W122R mutation, all affected members had hearing impairment and some had branchial and preauricular anomalies. Renal anomalies were not spontaneously reported in any affected individuals nor detected in three individuals analysed by ultrasound (Table 1).

Preliminary clinical experience based on the six-unrelated families with *SIX1* mutation suggests mandatory presence of hearing impairment, a variable presence of EVA, low frequency (25%) of branchial arch malformations, lacrimal duct stenosis in about 10% and absence of renal pathology<sup>10,17,28,29</sup> (see also this study). One patient in our family 2 (individual IV:5; Figure 1b) underwent a CT scan before cochlear implant, but no malformations of the temporal bones were found. The frequent occurrence of temporal bone malformations in BOR patients in previous reports<sup>7,8,30</sup> did not include verified mutations, but one study described a patient with a *SIX1* mutation.<sup>10</sup> The limited number of reported *SIX1* mutations, so far, the low frequency of renal and branchial malformations in individuals who underwent examination for these abnormalities and lack of identified *SIX1* mutations in eight Japanese BO patients may all indicate clinical differences in BO(R) patients with *EYA1* and *SIX1* mutations, respectively. However, more patients need to be studied in order to clarify this possibility.<sup>10</sup> *SIX1* mutations may be more prevalent in patients with hearing impairment and EVA, not caused by mutations in *SLC26A4*, than recognized so far. Therefore, we encourage more *SLC26A4*-negative patients with EVA and hearing impairment to be investigated for *SIX1* mutations, in order to establish better genotype-phenotype correlations in clinically different patients.

Three of the four *EYA1* mutations identified in this study (c.920delG, IVS10-1G>A and IVS12+4A>G) are predicted to lead to a protein lacking or with a disrupted phosphatase domain, (encoded by exons 9-16), and

thereby in agreement with previous reports.<sup>3,16,31</sup> Mutations in the phosphatase region are expected to lead to a non-functional protein, which is unable to bind to *SIX1* and *DACH*. The mutation, c.920delG in exon 8, leads to a frameshift at codon 307, and thereby a protein lacking the phosphatase domain. Both splice mutations, IVS10-1G>A and IVS12+4A>G, were predicted to cause non-functional *EYA1* protein with a disrupted phosphatase domain. The IVS10-1G>A splice mutation was previously reported by Chang *et al*<sup>3</sup> in a BOR patient. This, together with the perfect co-segregation of the same mutation in our large family 3, provides strong evidence that the mutation is pathogenic. The only clinical difference was the absence of reported renal anomalies in the patient described by Chang *et al*,<sup>3</sup> in contrast to our family 3 (Table 1), where 3 out of 7 analysed patients had renal pathology. Modifying factors, therefore, are suspected to play a role in determining the clinical implications of each mutation. Our family 5, previously reported with a main focus on ossicular chain abnormalities, hypoplastic cochleae and hypoplastic middle ear cavity, is now shown to be due to an *EYA1* mutation (IVS12+4A>G).

The novel nonsense mutation in *EYA1*, c.1773C>G (p.Y591X) in exon 16 in family 1 is located just outside the phosphatase domain. Interestingly, Rickard *et al*<sup>31</sup> identified a mutation (c1779A>C; X593Y(+5AA)) in the plus two codon relative to Y591 in a familial case (family 8 in Rickard *et al*<sup>31</sup>), and another nearby mutation (c.1748T>C; L583P) in a sporadic case (case 11 in Rickard *et al*<sup>31</sup>). The absence of our c.1773C>G mutation in 356 control chromosomes and the evolutionary conservation of this region in several species and conservation in *EYA1-4* also point to a functional role of Y591 and that the Y591X mutation is pathogenic.

The absence of *EYA1* mutation in family 4 is a puzzle considering the linkage results in favour of *EYA1* with a two-point LOD score of 2.32 increasing to 2.71 if the hypothesis of a *de novo* mutation in the probands mother (Figure 1e, III:5) is taken into account. Some investigators have previously suggested that mutations in *NDUFB9*, in the same region (8q13), could cause a BOR phenotype;<sup>32</sup> however, they did not find any *NDUFB9* mutations in nine BOR families. Another explanation might be a mutation in *EYA1* regulatory elements (conserved-nongenic-elements) close to *EYA1*, leading to an inactivation of *EYA1* transcription. There does not seem to be any CpG island near *EYA1*; hence methylation differences are not likely. Mutations in non-coding exons in *EYA1*, which could be involved in gene regulation, might be another explanation, but the normal sequence results for the non-coding exons did not support this possibility in family 4.

Our report adds to the accumulating genotype-phenotype correlations in BOR. The figures in Table 1 are minimum estimates regarding subtle and subclinical findings, because we only collected already available information

about renal and temporal bone abnormalities in these families. The importance of clinical evaluation and molecular testing in BOR has been shown by a number of authors testing both isolated and familial cases.<sup>31</sup> The clinical presentation of SIX1-related disease may even mimic non-syndromic hearing impairment, as illustrated in the DFNA23 family, who turned out to have SIX1 mutation and mild BOR pathology.<sup>17,29</sup> The detection rate of mutations in EYA1 and SIX1 in 5 out of 6 probands in this study are encouraging for sequencing EYA1 and SIX1 in clinically suspected patients, irrespective of a positive family history.<sup>31,33</sup> The diagnosis of Potter's syndrome in newborn, as illustrated by individual II:3 in family 6 (Figure 1d), should also elicit a suspicion of BOR and sequencing of EYA1 and SIX1.

Recently, we reported another Danish family with BOR syndrome caused by a novel splice site mutation (IVS9 + 1G > C) in EYA1.<sup>25</sup> In total, we identified an EYA1 or a SIX1 mutation in 6 out of 7 (86%) Danish BOR families by direct sequencing (see this study and Henriksen *et al*<sup>25</sup>). Our detection rate of mutations is comparable to the 71% reported in a series of Japanese patients (EYA1 mutation in 5 out of 7 patients, 2 familial and 5 sporadic, but no SIX1 mutations), also applying direct sequencing of both BOR genes<sup>33</sup> and the 61% in 18 patients (11 familial and 7 sporadic) in an English study using SSCP and sequencing of EYA1 only.<sup>31</sup> Several previous studies reported a much lower detection rate of EYA1 mutations of 25–40% in BOR patients with the highest success rate in familial cases.<sup>3,15,34,35</sup> The absence of EYA1 abnormalities detectable by MLPA, which was applied for the first time in the present study, does not support the previous suggestion of a 20% frequency of complex rearrangements of EYA1 as causative in BOR.<sup>15</sup> More reliable figures for the proportion of patients having deletions or duplications, detectable by MLPA, and the proportion having BOR due to SIX1 mutations can only be reached by studying more patients.

In conclusion, we identified disease causing EYA1 and SIX1 mutations in 5 out of 6 Danish families comprising 66 patients, and strongly encourage to look for mutations in clinically suspicious cases. A novel SIX1 mutation adds to the few cases reported so far. Our data provide substantial molecular and clinical information for establishing a better genotype–phenotype understanding of BOR. Larger studies including patients with hearing impairment and EVA, but no branchial or renal malformations, will clarify if the preliminary experience of different clinical picture of SIX1- and EYA1-related disease is consistent.

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