Mutation screening of the EYA1, SIX1 and SIX5 genes in a large cohort of patients harboring branchio-oto-renal syndrome calls into question the pathogenic role of SIX5 mutations

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Mutation screening of the *EYA1*, *SIX1* and *SIX5* genes in a large cohort of patients harboring branchio-oto-renal syndrome calls into question the pathogenic role of *SIX5* mutations

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ABSTRACT

Branchio-oto-renal (BOR) syndrome is an autosomal dominant disorder characterized by branchial, ear and renal anomalies. Over 80 mutations in *EYA1* have been reported in BOR. Mutations in *SIX1*, a DNA binding protein that associates with EYA1, have been reported less frequently. One group has recently described 4 missense mutations in *SIX5* in 5 unrelated patients with BOR.

Here, we report a screening of these three genes in a cohort of 140 patients from 124 families with BOR. We identified 36 *EYA1* mutations in 42 unrelated patients, 2 mutations and one change of unknown significance in *SIX1* in 3 unrelated patients, but no mutation in *SIX5*. We did not find correlation between genotype and phenotype, and observed a high phenotypic variability between and within BOR families. We show the difficulty in establishing a molecular diagnosis strategy in BOR syndrome; the screening focusing on patients with typical BOR would detect a mutation rate of 76%, but would also miss mutations in 9% of patients with atypical BOR. We detected a deletion removing three *EYA1* exons in a patient who was previously reported to carry the *SIX5* Thr552Met mutation. This led us to reconsider the role of *SIX5* in the development of BOR.

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INTRODUCTION

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Branchio-oto-renal (BOR) syndrome is an autosomal-dominant developmental disorder which is characterized by hearing loss, branchial arch defects and various renal anomalies. The prevalence of BOR syndrome is estimated to be 1 case per 40 000 (Chen et al., 1995; Fraser et al., 1978; Fraser et al., 1980, Melnick et al., 1975, Melnick et al., 1978). The syndrome is clinically heterogeneous and has a high penetrance with variable expressivity (Fraser et al., 1978, Fraser et al., 1980, Chen et al., 2004). BOR syndrome is also genetically heterogeneous. Over 80 mutations in *EYA1* (MIM ID 601653), the human homolog of the *Drosophila eyes absent* gene, encoding a transcriptional regulator, have been identified. These include large and small heterozygous deletions, frameshift, stop, splice-site and missense heterozygous mutations (Abdelhak et al., 1997b, Ni et al., 1994, Vincent et al., 1997). The rate of detection of *EYA1* mutations varies from 7% to 40% of patients tested according to the clinical criteria required for molecular testing (Abdelhak et al., 1997a, Abdelhak et al., 1997b, Chang et al., 2004, Orten et al., 2008). Mutations in *SIX1* (MIM ID 601205) (mainly missense mutations and small deletions), the human homolog of *sine oculis* encoding a DNA binding protein that associates with EYA1, have also been associated with BOR syndrome (Kochhar et al., 2008, Ruf et al., 2003, Ruf et al., 2004, Sanggaard et al., 2007), though much less frequently than *EYA1* mutations. More recently, missense mutations in another *SIX* family member, *SIX5* (MIM ID 600963), have been reported by one group in patients with BOR syndrome (Hoskins et al., 2007). SIX5 homologous is known to interact with eya-1 in *C. elegans*. *In vitro* functional analyses of the BOR-associated *SIX5* variants showed that some of these variants modified EYA1-SIX5 binding and the ability of the EYA1-SIX5 complex to transactivate a

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reporter gene (Hoskins et al., 2007). However, the association of *SIX5* mutations with BOR syndrome has not been confirmed by other groups.

In the present study, we screen for *EYA1*, *SIX1* and *SIX5* mutations a large cohort of patients with BOR syndrome. We describe the clinical features associated with the mutations and the rate of mutations identified, according to the clinical phenotypes. We also show that one family previously reported as carrying a *SIX5* missense mutation harbors a heterozygous deletion of three *EYA1* exons, which therefore questions the role of the reported *SIX5* change.

PATIENTS AND METHODS

Patients

A total of 140 patients from 124 families with a diagnosis of BOR syndrome were included in the study. Subjects were classified according to the criteria defined by Chang (Chang et al., 2004) on the basis of clinical history, audiometry and renal ultrasonography. Patients were considered as typical BOR when they had at least three major criteria (branchial anomalies, deafness, preauricular pits or renal anomalies), or two major and two minor criteria (internal, middle and/or external ear anomalies, preauricular tags, facial asymmetry or palatine anomalies) or one major criterion and an affected first-degree relative meeting the above criteria for typical BOR. Other were considered as atypical BOR and were tested only when they demonstrated at least two features of the syndrome.

Patient 1062 was previously reported as carrying a heterozygous *SIX5* c.1655C>T (p.Thr552Met) mutation (patient A500 in Hoskins et al.). His DNA had been tested for *EYA1* mutations by direct sequencing, but not for abnormal copy number (Hoskins et al., 2007). This patient was having assisted reproduction, and thus was making inquiries regarding the possibility of preimplantation genetic testing.

Patients' samples, medical records, genealogy and written informed consent from patient and/or parents were sent from Paediatric, Paediatric Nephrology, Nephrology, or Genetics departments between August 2004 and December 2009.

Mutation analysis

Genomic DNA was isolated from peripheral blood using standard methods. The 16 exons of *EYA1* were screened for mutations by direct sequencing. When no mutation was found, quantitative multiplex PCR amplification of short fluorescence fragments (Charbonnier et al., 2000) was performed for *EYA1* exons 1, 5, 10, 15 and 16. When a deletion was found to remove some but not all of the exons tested, the DNA sample was analysed by multiplex ligation dependent probes amplification (Salsa MLPA kit P153 EYA1, MRC-Holland, Amsterdam, Netherlands). When neither mutation nor deletion was found in *EYA1*, the 2 exons of *SIX1* and the 3 exons of *SIX5* were screened by direct sequencing. For previously unreported missense mutations, 92 control individuals were tested by direct sequencing. The cDNAs NM_172060.2 for *EYA1*, NM_005982.3 for *SIX1* and NM_175875.4 for *SIX5* were used for numbering, with nt +1 corresponding to the A of the ATG translation initiation codon. One DNA sample (patient 608), was shown to have large 8q13.3 deletion by FISH analysis and was used as a positive control for deletion screening. Missense mutations were evaluated using the softwares PolyPhen (http://genetics.bwh.harvard.edu/pph/) and ConSurf (http://consurftest.tau.ac.il/).

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Statistical tests

Testing for difference in proportions was performed using either the X^2 or Fisher's exact test. All tests were two sided. *P*-values <0.05 were considered significant.

RESULTS

Mutations

The mutations identified and the associated phenotypes are shown in table 1. In the entire cohort (140 patients from 124 families) we identified 36 *EYA1* mutations in 42 families (55 patients), two *SIX1* mutations and one *SIX1* variant of unknown significance in 3 families (4 patients), but no *SIX5* mutation. We identified two *SIX5* variants which were not considered to be responsible for the phenotype: one was found in controls, and the other, previously reported as a disease causing mutation in two families (Hoskins et al., 2007), was associated with a partial *EYA1* deletion in one of these two families included in our cohort. Therefore, mutations were identified in 36% (45/124) of the tested families.

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EYA1 gene analysis

Thirty-six *EYA1* heterozygous mutations, spread over the entire length of the gene (figure 1), were identified in 42 probands (table 1). Thirty-three were small mutations (8 missense including a mutation of the stop codon, 14 frameshift, 6 stop, and 5 splice-site mutations), and 24 of these were novel. All missense mutations but one [c.319G>A (p.Gly107Ser)] were considered as possibly or probably damaging by the PolyPhen software (http://genetics.bwh.harvard.edu/pph/). That mutation c.319G>A, which appeared *de novo* in the patient, was scored as benign by Polyphen but was in the last base of exon 4 and thus was expected to modify the splicing of intron 4 (GeneSplicer score changes from 6.97 to 2.49) . Amino acid conservation scores according to ConSurf (varying from 1 to 9) for previously unpublished missense mutations are shown in table 1.Two previously reported mutations, c.982C>T and c.1220G>A, were respectively found in three and two unrelated patients. Three different deletions were identified in 5 unrelated patients by quantitative multiplex PCR amplification of short fluorescence fragments. In 3 of these probands (patients 608, 821, 991) all tested exons were missing and the deletion was considered to remove the entire gene. In

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the other two cases, the deletion was partial and its precise extent was determined by MLPA analysis: one removed exons 8 to 16 (patient 1216) and one removed exons 3, 4 and 5 (patients 1062, 1063 and 1064). Patient 1062 was one of the 2 probands previously reported to carry the *SIX5* p.Thr552Met mutation (patient A500 in Hoskins, et al., 2007). Parent status was tested for 26 probands with an identified *EYA1* mutation : 8 mutations out of 26 were *de novo* and 18 were inherited.

SIX1 gene analysis

Three different, potentially pathogenic variations in *SIX1* were identified in 3 families (table 1). The *SIX1* mutation (p.Tyr129Cys) has already been described (Ruf et al., 2004), and affects a conserved tyrosine in the homeodomain. It is predicted to be probably damaging by Polyphen (score 2.945), and inhibits the transcription activation *in vitro* (Patrick et al., 2009). This mutation was also present in the affected father of the proband. The mutation c.560+3A>T, probably leading to aberrant splicing (GeneSplicer score changes from 9.74 to 2.89), has never been previously described. Finally, the c.746C>T change in exon 2 affects a strongly conserved amino acid (p.Pro249Leu) and was considered as possibly damaging by PolyPhen (score 1.806). Although this change was not present in 92 healthy control chromosomes it is located in a region of unknown function and replaces a non polar side chain amino acid with another amino acid of the same family. The segregation of these two last changes could not be tested because DNA samples from family members were not available.

SIX5 gene analysis

We did not identify any novel *SIX5* mutations in our entire cohort. We confirmed the finding of the *SIX5* p.Thr552Met heterozygous variant in patient 1062, and also found the same *SIX5* variant in his twin brother (patient 1063) and in his father (patient 1064), who were both affected. This variant was predicted as possibly damaging by the Polyphen program (score 1.711). However, we identified an *EYA1* partial deletion in the three affected members of this family (see above). We found another *SIX5* variant, c.156_161dup (p.Gly55Ala56dup), in a patient from Guadeloupe. That variant, which introduces two amino acids in the N-terminus of the protein, was also found in 3/86 controls from the West Indies and thus was considered as non pathogenic.

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Phenotypes (tables 1 and 2)

According to previously described criteria (Chang et al., 2004), our population included 67 patients with typical BOR and 55 patients with atypical BOR. In 18 cases the information we had was insufficient to classify patients as typical or atypical. Sixty eight probands had a family history of BOR (38 typical cases of BOR and 30 cases of renal anomaly, branchial arches defects and/or deafness, which did not fulfill the typical BOR diagnosis criteria). The phenotypic features of patients with an identified mutation are detailed in table 1.

The frequency of each symptom in our entire population as well as in patients with mutation and in patients without mutation is summarized in table 2. The various renal phenotypes observed in patients with an identified mutation are described figure 2. The renal function of these patients varied greatly, from a normal glomerular filtration rate to end-stage renal failure. In four cases prenatal renal failure diagnosed by oligoamnios during the second trimester of pregnancy was observed in fetuses displaying kidney hypoplasia (patients 175, 700, 991 and 1126), and this led to termination of pregnancy in three of these cases. Five patients received a renal transplant: two reached end stage renal failure during childhood (at 14 and 16 years), one at 26 years, and two others at unknown age (but one received a transplant at 22). In some families (see cases 700 and 1126 as examples), although a fetus or a

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child had severe renal disease, the affected parent had normal renal function and normal kidneys on ultrasound examination. After renal disease, the most frequent symptom was deafness (conductive and/or sensorineural), followed by pits, branchial defect and then by ear tags.

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Some patients presented with rare phenotypic features. In patients with an identified mutation, two (patients 311 and 1291) presented with cataract (unilateral in one case and bilateral in the other). Five patients had facial nerve impairment: unilateral facial paralysis, crocodile tears syndrome and facial asymmetry. Palate anomalies were observed in 5 patients: short palate responsible for hypernasal speech, arched palate, palatine cleft, bifid uvula and posterior agenesia of uvula. We observe two heart defects: a persistent ductus arteriosus in patient 708 and a conotroncal cardiopathy (pulmonary atresia with interventricular communication) in patient 700. Finally, patient 229 suffered from hypothyroidism of unknown etiology.

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In patients without any identified mutation, rare clinical features were also observed : palate or laryngeal anomalies (n=4), facial asymmetry (n=2), bifid uterus (n=2), interventricular communication (n=2), clinodactyly (n=1), aortic arch hypoplasia (n=1), cutaneous pigmentation anomalies (n=2), Malherbe’s calcifying epithelioma (n=1), dorsal medullar atrophia responsible for pyramidal syndrome (n=1), pulmonary hypertension of unknown etiology (n=1), cerebral ventricular dilation (n=1), thyroglossal duct cyst (n=1), jejuno-ileal atresia (n=1), and bone anomalies (radial aplasia, mandibular hypoplasia, dental agenesis, postaxial polydactyly) in four patients.

Genotype-phenotype correlation

Because the type and severity of the symptoms were very variable, we searched whether there was a correlation between the phenotype and either the mutated gene (*EYA1* or *SIX1*) or the type of mutation (missense mutation, truncating mutation because of stop, frameshift, or splice-site mutation), or deletion. Of the 67 patients with typical BOR syndrome, 50 (75%),

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had a mutation in *EYA1*, and one had a change in *SIX1* of unknown significance. Of the 55 patients with atypical BOR syndrome, 5 (9%) had a mutation (in *EYA1* in 4 cases and in *SIX1* in 1 case). The proportion of patients affected with typical or atypical BOR syndrome was not significantly different (p=0.38) between patients carrying a missense mutation (9/11 typical), a truncating mutation (34/37 typical) or a deletion (8/8 typical). The same mutation (p.Arg328X) was identified in a patient with atypical BOR and in 2 patients with typical BOR. We did not observed any particular phenotypic features associated with *SIX1* mutations. Rare features were observed both in patients carrying *EYA1* or *SIX1* mutations (assuming that the *SIX1* p.Pro249Leu is responsible for the phenotype). Among the 55 patients with *EYA1* mutations, the proportion of deletion (n=9), missense (n=10), and truncating (n=36) mutations was not significantly different between patients with (n=40) or without (n=15) deafness (p=0,46), with (n=30) or without (n=25) kidney involvement (p=0.35), with (n=32) or without (n=23) pits (p=1), or with (n=34) or without (n=11) branchial defect (p=0.66) (Table 3). Regarding the renal disease, cases with prenatal renal failure associated with oligoamnios (n=4) or with severe renal failure leading to renal transplant (n=5) were associated with *SIX1* mutations in one case, and with *EYA1* mutations in 7 cases. These proportions were not significantly different from that observed in all patients. However, in the 7 cases with *EYA1* mutations, none of these mutations were a missense mutations (3 were frameshift, 1 splice-site, 1 stop and 2 were entire gene deletions).

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DISCUSSION

To our knowledge, this study is the first one to analyze all of the genes currently known to be implicated in BOR syndrome in a large cohort of patients. Overall, we detected a mutation in 45/124 (36%) probands. Forty two probands were carrying an *EYA1* mutation thought to be pathogenic. However, the impact of the c.867+5G>A change on mRNA splicing has not yet been demonstrated. Three probands were carrying mutations in *SIX1*. Of these, one (patient 714) was carrying the c.806C>T, p.Pro249Leu change, which has never been described before, and is predicted to be possibly damaging by Polyphen. This mutation was not found in 92 control chromosomes, but does not affect a protein domain with a known function. All other *SIX1* mutations reported so far affect either the SIX or the homeodomain encoding nucleotides (Kochhar et al., 2008, Ruf et al., 2004). We were unable to test other family members of patients 714 and 715, so it is difficult to conclude whether this change is or not a disease-causing mutation.

In our entire cohort, we did not identify any pathogenic mutation in the *SIX5* gene. In one patient previously reported to carry a *SIX5* missense variant (case 1062), we found a partial (exons 3-5) *EYA1* deletion. The three affected patients in this family were carrying both the *EYA1* deletion and the *SIX5* variant. We believe that the *EYA1* deletion is responsible for the phenotype in this family, though we cannot rule out the hypothesis that the *SIX5* variant may modify the *EYA1*-associated phenotype. However, whereas the three patients had deafness, the renal disease was more severe in the two siblings (undergoing renal transplantation at 22 and 23 years of age) than in their father (who had not reached end-stage renal failure at 58 years). This was despite the fact that all three carried the *SIX5* variant. The finding of an *EYA1* mutation in that family made us reconsider the role of *SIX5* in the development of BOR syndrome. Among the 5 index cases reported by Hoskins et al. as carrying a *SIX5* mutation (Hoskins et al., 2007), all carried a missense variant, including two cases with the c.1655C>T p.Thr552Met variant (patient 1062 and another patient). The segregation of the variants with the phenotype had not been studied. These variants modestly (20 to 48%) although significantly decreased the ability of SIX5/EYA1 to activate gene transcription *in vitro* (Hoskins et al., 2007). No other *SIX5* mutation (whether missense or other type of mutation/rearrangment) has been reported since this initial report. In addition, whereas mice

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with *Six1* or *Eya1* gene inactivation develop ear and kidney abnormalities, the phenotype in *Six5*-/- mice is limited to abnormalities in the eye (cataract), and does not affect the ear or the kidney. Taken together, these findings strongly suggest that *SIX5* mutations are not directly responsible for BOR syndrome.

Among the patients that were classified with either typical or atypical BOR (122 patients), we identified a mutation in 75% of cases with typical BOR syndrome and in 9% of cases with atypical BOR syndrome. These results are different from those reported recently in a smaller cohort in which no mutations were detected in any subject with atypical BOR (Rickard et al., 2008). This highlights the difficulty in reconciling the need for performing molecular testing in a consequential and cost effective manner, and the fact that a screening limited to typical BOR syndrome will miss few mutations and prevent accurate genetic counselling in these few families. The rate of mutation that we report here is not different from that recently reported in another large cohort (Orten et al., 2008). However, the rate of *EYA1* deletion in the present study is lower than that (18%) reported by Chang et al. (Chang et al., 2004). Although we used the same semiquantitative fluorescence multiplex PCR approach for tracking *EYA1* deletions, we only tested exons 1, 5, 10, 15, and 16 in a first attempt. We may have thus missed small or complex deletions involving other exons.

In our series as in others (Chang et al., 2004, Ruf et al., 2004, Saanggard et al., 2007, Okada et al., Orten et al., 2008), the type and severity of the phenotype does not seem to correlate with the type of mutation and is very variable, even within a given family. Only the severity of renal failure may correlate to some extent with the type of *EYA1* mutation, as none of the 7 patients with the most severe renal insufficiency were carrying a missense mutation. However the small number of patients does not allow any conclusion to be made, and it would be interesting to analyze the severity of the renal failure in a larger number of cases carrying an *EYA1* mutation. The high frequency of renal anomalies in our series may be due to the fact that our laboratory is focused on renal diseases. The important phenotypic variability observed in our cohort as in others may be due to genetic and/or environmental factors (in particular maternal factors during embryonic and fetal development) that modify the phenotype. This makes genetic counselling particularly difficult for these families.

We report some interesting clinical features associated with *EYA1* mutations. Although already reported by others (Chen et al., 1995, Shimasaki et al., 2004) the association with hypothyroidism or with persistent ductus arteriosus may be fortuitous, as may be the association with a conotruncal cardiopathy. Two patients suffered from cataract, which could be associated with a defect of early expression of *EYA1* in the anterior ocular segment, and has already been described (Azuma et al., 2000). A facial nerve impairment was present in six patients, which may be explained by anomalies of inner ear, affecting the nerve trajectory.

In conclusion, our results confirm that *EYA1* is by far the most frequently mutated gene in BOR syndrome today and seriously question the role of *SIX5* variants in the pathophysiology of BOR syndrome. They confirm the lack of genotype-phenotype correlation and illustrate the difficulty in establishing an algorithm for molecular diagnosis in BOR syndrome. The screening of patients with typical BOR only would greatly increase the rate of identified mutations but would also lead to missed mutations in a few families. Our data suggest that testing patients with atypical BOR still results in the identification of a few mutations, but screening of the *SIX5* gene can be given up.

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Figure legend

Figure 1 : Schematic representation of *EYA1* gene. Boxes represent *EYA1* exons. Mutations: frameshift ✦, nonsense ●, missense ⚹, splice site ⯌, deletion •••••

EyaHR: eyes absent homologous region.

Figure 2: Renal phenotypes in patients with an identified mutation.

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<sp>Figure 2 : Pedigree of patient 1064 and his twin sons (patients 1062 and 1063). . . <sp> . . ¶
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Patient	Family history	Phenotype		Rare features	Gene	Exon or IVS	Nucleotide change	Protein change (conservation score**)	Polyphen	Mutation inheritance	Reference
346		T	b, f		*EYA1*	4	c.184C>T	p.Pro62Ser* (5)	Possibly damaging Score 1,851	I	this study
673		U	b, d (r : U)		*EYA1*	4	c.303C>A	p.Tyr101X	-	N	BTNRH, unpublished
383		T	b, p, r	Language delay	*EYA1*	4	c.319G>A	p.Gly107Ser* (7)	Benign Score 0,456	N	this study
656		T	d, p, r, f		*EYA1*	5	c.430C>T	p.Gln144X	-	U	this study
1126	1126 patient's father	T	r, f, TOP	Potter's sequence	*EYA1*	7	c.586_596Dup (+)636_644delInsTG	p.Ser200IlefsX12	-	I	this study
1318		T	b, d, f							U	
1153		T	d, p, r, ie		*EYA1*	7	c.616dupT	p.Tyr206LeufsX50		U	this study
708		T	b, p, ee (d : U)	Lacrymal duct stenosis, bifid uvula, persistent ductus arteriosus	*EYA1*	7	c.670delC	p.Gln224SerfsX109	-	U	this study
311		T	d, p, ee, t	Cataract, facial asymmetry	*EYA1*	7	c.722delC	p.Thr241LysfsX92	-	I	this study
523		T	d, p, ee, f (r : U)		*EYA1*	8	c.781C>T	p.Arg261X	-	U	Kumar et al, 1998
1215	BOR in her mother	T	b, r, ee, f							U	
326		T	b, p, f (d and r : U)		*EYA1*	8	c.783delA	p.Leu262CysfsX71	-	I	this study
327	326 patient's mother	T	b, d, f							U	
1291		A	b, p	Bilateral cataract	*EYA1*	IVS8	c.867+5G>A	-	-	U	this study
1311		T	b, d, p, ee	Facial paresia	*EYA1*	8	c.867_867+14del	p.Arg290GlufsX43	-	U	this study
314		T	b, d, p, ie, f	Epicanthus, hypernasal speech	*EYA1*	IVS9	c.952-2A>G	-	-	I	Okada et al, 2006

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117		T	b, d, r		*EYA1*	10	c.982C>T	p.Arg328X	-	U	BTNRH, unpublished
332		A	r, ee (d : U)	Dysmorphic features, arched palate, unilateral ovarian agenesis						U	
347		T	b, p, r, ee							N	
512		T	b, d, p, r, ee, ie, f	Motor delay, cleft palate, lacrymal duct agenesis	*EYA1*	10	c.989A>T	p.Glu330Val* (9)	Probably damaging Score 2,568	I	this study
417	512 patient's mother	T	b, d, r, ee, ie, f							U	
1056		T	b, p, ee, ie, t, f		*EYA1*	10	c.1039G>T	p.Glu374X	-	I	this study
1057	1056 patient's father	T	d, p, ee, f	Facial asymetria					-	U	
1282		T	b, d, p, r		*EYA1*	IVS 10	c.1042-1G>A	-	-	U	MORL
1288		T	b, d, p, r		*EYA1*	IVS 11	c.1100+1G>C	-	-	U	this study
693		T	b, d, r, ee, ie		*EYA1*	12	c.1216_1219dup	p.Arg407GlnfsX13	-	U	this study
1194		T	b, p, r, ee, t	Crocodile tears syndrome	*EYA1*	12	c.1220G>A	p.Arg407Gln (5)	Possibly damaging Score 1.766	U	Kumar et al, 1997
1202	BOR in his father	T	b, d, f							U	
1321		T	b, d, r, ee		*EYA1*	12	c.1231_1232dupAT	p.Tyr412SerfsX24	-	U	this study
780		T	b, p, f		*EYA1*	12	c.1251delinsCC	p.Asn418GlnfsX10	-	I	Abdelhak et al, 1997
781		T	b, d, p, ee, f							I	
782	780 and 781 patients' mother	T	b, d, p							I	
783	782 patient's grand-mother	T	d, f							U	
285		T	b, d, p, r, ee, ie, f		*EYA1*	13	c.1372_1375dupTCCC	p.Arg459LeufsX41	-	N	this study
286	285 patient's mother	T	b, d, p, r, f							U	
175		T	r, ee, f, (d : U), TOP	Fetal hypotrophia	*EYA1*	IVS13	c.1377-2A>G	-	-	I	BTNRH, unpublished
548	900 and 700 patients' father	T	b, d, p, f		*EYA1*	14	c.1425delA	p.Leu476TrpfsX9	-	U	this study
900		T	d, p, f							I	
700		T	r, f, TOP	Conotroncal cardiopathy						I	

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710		T	b, p, r	Uvula agenesis	*EYA1*	14	c.1434dup	p.Val479SerfsX20	-	N	this study
497		A	p, d, ee	Crocodile tears syndrome	*EYA1*	14	c.1442T>C	p.Leu481Pro* (8)	Probably damaging Score 2,517	N	this study
229		T	p, d, ie, f	Hypothyroidism	*EYA1*	14	c.1481A>G	p.Tyr494Cys (8)	Probably damaging Score 3,048	U	BTNRH, unpublished
148		T	b, d, p, r, ie, t		*EYA1*	15	c.1542_1546delAAAAG	p.Arg514SerfsX83	-	U	this study
953		T	b, d, r	Lacrymal duct stenosis	*EYA1*	15	c.1554T>G	p.Tyr518X	-	U	this study
1014		A	d, t		*EYA1*	16	c.1607T>C	p.Met536Thr* (9)	Probably damaging Score 2,723	U	this study
483		T	d, p, r, ee, ie		*EYA1*	16	c.1655dup	p.His552GlnfsX47	-	I	this study
1265		T	d, p, r		*EYA1*	16	c.1678T>C	p.X560Gln*	-	U	this study
1062		T	b, d, r, t, f		*EYA1*	3, 4 and 5	c.104- ?_461+?del	-	-	I	this study
1063	1062 patient's brother	T	d, r, f							I	
1064	1062 and 1063 patients' father	T	b, d, t, r, f							U	
1216		T	b, d, p (r : U)		*EYA1*	8-16	c.727-?_1680+?del	-	-	U	this study
608		T	b, d, p, r, ee	Cutaneous mastocytosis, hypoplasia of the triangular muscle of the lip, hypermetropia	*EYA1*	-	Entire gene deletion	-	-	N	
821		T	b, d, r, ee, t, f	Micrognathia, feeding troubles, central and obstructive apnea						I	
798	821 patient's mother	T	b, d, ee, f							U	
991		T	b, d, p, r	Facial asymmetry						N	
714		U	b (d : U)		*SIX1*	1	c.386A>G	p.Tyr129Cys	Probably damaging Score 2,945	I	Ruf et al, 2004
715	714 patient's father	U	b, ee (d : U)							U	

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1226		A	b, p		*SIX1*	IVS 1 + 3	c.560+3A>T	-	-	U	this study
162		T	r, f	Lacrymal duct stenosis	*SIX1*	2	c.746C>T	p.Pro249Leu* (7)	Possibly damaging Score 1,806	U	this study

Table 1 : mutations and phenotypes in patients with BOR syndrome. : T : typical BOR, A : atypical BOR, b : branchial anomalies, d : deafness, p : pits, t : tags, ie : inner ear, me : middle ear, ee : extern ear anomalies, r : renal anomalies, f : familial, U : unknown, TOP : termination of pregnancy, I : inherited, N : de novo. BTNRH and MORL : Boys Town National Research Hospital and Molecular Otolaryngology Research Laboratory (UI, USA), see http://www.healthcare.uiowa.edu/labs/pendredandbor/. * : not found in 92 healthy individuals. ** :according to ConSurf (http://consurftest.tau.ac.il/). The reference sequences for numbering are NM_172060.2 for *EYA1*, NM_005982.3 for *SIX1* and NM_175875.4 for *SIX5* .

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	Total		With a mutation		No mutation	
	n	%	n	%	n	%
Renal anomalies	89	65%	31	53%	58	72%
Deafness	88	64%	40	68%	48	59%
Pits	56	41%	33	56%	23	28%
Branchial defects	55	40%	37	63%	18	22%
Tags	31	23%	8	14%	23	28%

Table 2 : clinical features of the whole population (n=140). Columns with and without mutation represent the ratio of patients with each symptoms reported on the total number of patients with (n=59) or without (n= 81) mutation, respectively

	Deafness	No deafness	Total
Missense	8	2	10
Truncating	24	12	36
Deletion	8	1	9
	40	15	55

	Renal symptoms	No renal symptom	Total
Missense	5	5	10
Truncating	18	18	36
Deletion	7	2	9
	30	25	55

	Pits	No pit	Total
Missense	6	4	10
Truncating	22	14	36
Deletion	4	5	9
	32	23	55

	Branchial symptoms	No branchial symptom	Total
Missense	6	4	10
Truncating	22	14	36
Deletion	6	3	9
	34	11	55

Table 3: type of *EYA1* mutation according to the type of symptom

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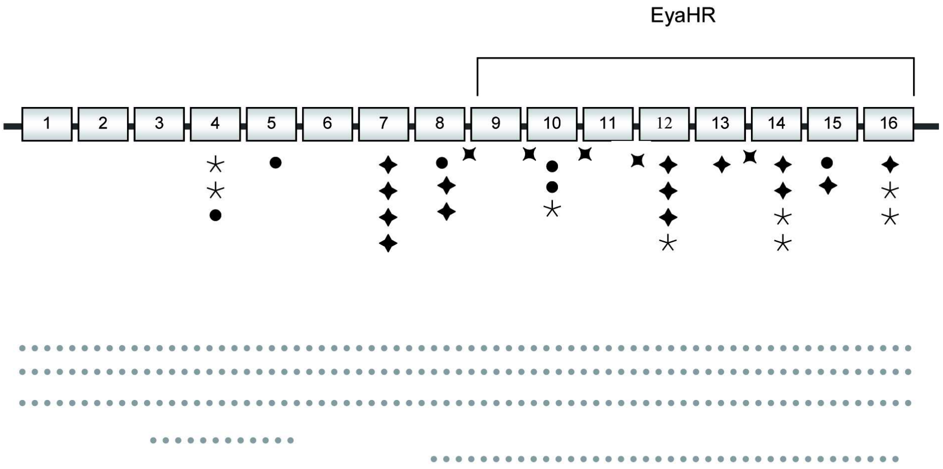

Schematic representation of EYA1 gene. Boxes represent EYA1 exons. Mutations: frameshift ☐, nonsense ☐, missense ☐, splice site ☐, deletion EyaHR: eyes absent homologous region.
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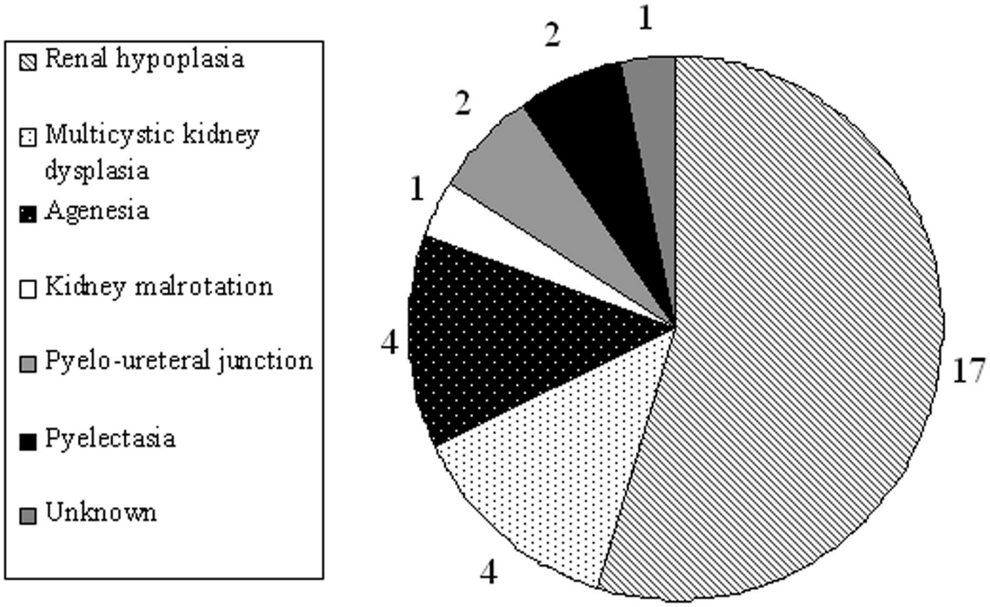

Renal phenotypes in patients with an identified mutation.
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