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[Original article]

Clinically diverse phenotypes and genotypes of patients with branchio-oto-renal syndrome

Running title: BOR syndrome in Japan

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Abstract

Branchio-oto-renal (BOR) syndrome is a rare autosomal dominant disorder characterized by branchiogenic anomalies, hearing loss, and renal anomalies. The aim of this study was to reveal the ~~relationships between~~ clinical phenotypes and their causative genes in Japanese BOR patients.

Patients clinically diagnosed with BOR syndrome were analyzed by direct sequencing, multiplex ligation-dependent probe amplification (MLPA), array-based comparative genomic hybridization (aCGH), and next-generation sequencing (NGS). We identified the causative genes in 38/51 patients from 26/36 families; *EYAI* aberrations were identified in 22 families, *SALL1* mutations were identified in two families, and *SIX1* mutations and a 22q partial tetrasomy were identified in one family each. All patients identified with causative genes suffered from hearing loss. Second branchial arch anomalies, including a cervical fistula or cyst, preauricular pits, and renal anomalies, were frequently identified (> 60%) in patients with *EYAI* aberrations. Renal hypodysplasia or unknown-cause renal insufficiency was identified in more than half of patients with *EYAI* aberrations. Even within the same family, renal phenotypes often varied substantially. ~~We did not identify any conclusive relationships between genotype and phenotype in BOR patients.~~ In addition to direct sequencing, MLPA and NGS were useful for the genetic analysis of BOR patients.

Keywords

Branchio-oto-renal syndrome, *EYA1*, renal anomaly, next-generation sequencing.

INTRODUCTION

Branchio-oto-renal (BOR) syndrome (BOR1 #113650, BOR2 #610896) is an autosomal dominant disorder characterized by branchiogenic anomalies, hearing loss, and renal disorders including congenital anomalies of the kidney and urinary tract (CAKUT).¹ Patients with symptoms similar to those of BOR syndrome, but without renal anomalies, are diagnosed with branchio-oto syndrome (BOS) (BOS1 #602588, BOS3 #608389). **BOR syndrome and BOS are spectrum disorders;**² **therefore, they are collectively referred to as BOR syndrome here.** BOR syndrome is a rare disorder; the prevalence of BOR syndrome is 1:40,000 births in European populations,²³ and approximately 250 Japanese patients were diagnosed with BOR syndrome in 2010.¹ The known causative genes for BOR syndrome are *EYA1* (8q13.3), *SIX1* (14q23.1) and *SIX5* (19q13.32).³² In addition, pathogenic variants of *SALL1* (16q12.1), the causative gene for Townes-Brocks syndrome (TBS, #107480), may display a BOR-like phenotype in affected patients.^{4, 5} *EYA1* is the most frequently identified gene for BOR syndrome;³² it is a transcription factor essential for the development of the kidney, as well as the first and second branchial arches. Patients with pathogenic variants of *EYA1* can present with various clinical phenotypes including and especially renal disease; renal disease is the most important prognostic factor for the patient's quality of life. Currently, no correlations between the genotype and phenotype of BOR patients have been identified. In this study, we investigated clinical phenotypes and their causative genes in Japanese patients with BOR syndrome.

MATERIALS AND METHODS

Ethics Statement

Genetic analyses were performed after obtaining written informed consent from all patients or their legal guardians. All procedures were reviewed and approved by the Institutional Review Board of the Kobe University School of Medicine (65 and 301), and were performed in accordance with the ethical standards established in the Declaration of Helsinki.

Patients

We analyzed patients clinically diagnosed with BOR syndrome in a Japanese population from September 2010 to September 2017. The diagnostic criteria for BOR syndrome followed those defined by Chang *et al.*⁶ Briefly, the major criteria were: 1) second branchial arch anomalies, including a cervical cyst (CC) and cervical fistula (CF); 2) hearing loss (HL); 3) preauricular pits (PP); 4) auricular deformities (external ear anomalies, EEA); and 5) renal anomalies. The minor criteria were: 1) external auditory canal anomalies (EACA); 2) middle-ear anomalies (MEA); 3) inner-ear anomalies (IEA); 4) preauricular tags (PT); and 5) facial asymmetry and palate abnormalities, among others. Patients without a family history were diagnosed with BOR syndrome if three or more of the above major criteria, or two major and at least two minor criteria, were

identified. If patients had a family history of BOR syndrome, they were diagnosed with BOR syndrome if even one major symptom was identified. We excluded patients with phenotypes characteristic of other syndromes; for example, moderate to severe intellectual disability (ID) or motor developmental delay (MDD), long palpebral fissures, preaxial polydactyly, or imperforate anus. These phenotypes were more likely to be caused by CHARGE syndrome (#214800), Kabuki syndrome (#147920, #300867), or TBS. In this study, we included patients with renal anomalies and mutations in BOR-related genes, as identified by next-generation sequencing (NGS). The characteristics of the patients are listed in Table 1, Table 2, and Supplementary Table 1.

Genetic analysis

The genomic DNA of patients was extracted from peripheral blood mononuclear cells using the QuickGene whole blood kit S (Kurabo, Osaka, Japan) according to the manufacturer's instructions. The genomic DNA of a deceased boy (the brother of SC19) was extracted from the dried umbilical cord using the QuickGene tissue kit S (Kurabo).

First, *EYAI* (accession no. NM_000503.4, NP_000494.2), *SIX1* (NM_005982.3, NP005973.1), *SALL1* (NM_001127892, NP_001121364), and *SIX5* (NM_175875.4, NP_787071.2) were analyzed by Sanger sequencing (Supplementary Table 2). All exons were amplified by PCR. The PCR products were purified and analyzed for the direct sequencing of all exons and their

boundary introns using the Dye Terminator Cycle Sequencing Kit (GE Healthcare, Little Chalfont, UK) with an ABI Prism 3130 system (Applied Biosystems, Foster City, CA, USA). For patients where a causative gene was not identified by direct sequencing, multiplex ligation-dependent probe amplification (MLPA) was performed using SALSA MLPA P153 EYA1 (for *EYA1*) and P180 Limb malformations-2 (for *SALL1*) probe mix according to the manufacturer's instructions (MRC-Holland, Amsterdam, Netherlands).

We performed array-based comparative genomic hybridization (aCGH) or NGS on five patients using the SurePrint G3 Human CNV microarray 400 K kit or the SurePrint G3 Human CGH microarray 180 K kit (Agilent Technologies, Santa Clara, CA, USA) for aCGH, according to the manufacturer's instructions. NGS was performed using the Illumina TruSight One (TS1) sequencing panel or HaloPlex HS on a MiSeq platform according to the manufacturer's instructions (Illumina, San Diego, CA, USA) as previously described.⁷ The gene list constructed in this study using HaloPlex HS can be found in Supplementary Table 3 (v. 2), 4 (v. 3), and 5 (v. 4). The variants were confirmed by Sanger sequencing.

Data analysis

Sanger sequencing data were analyzed using the CLC main workbench v. 6.7.1 (Qiagen, Hilden, Germany). The aCGH data were analyzed by CytoGenomics (Agilent Technologies). To analyze the

data from the TS1 sequencing panel, we used VariantStudio v. 2.1.46 (Illumina) and Integrative Genomics Viewer software v. 2.3.57 (Broad Institute, Cambridge, MA, USA). The data from HaloPlex and HaloPlex HS were analyzed by SureCall v3.5 (Agilent Technologies). The reference genome used was hg19. Missense variants were evaluated using PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.jcvi.org/>) and Mutation Taster (<http://www.mutationtaster.org/>). Splice site variants were evaluated using Human Splicing Finder (<http://www.umd.be/HSF3/>). The following databases were accessed using AlamutVisual v2.9.0 (Interactive Biosoftware, Rouen, France), the Human Gene Mutation Database (HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), ExAC (<http://exac.broadinstitute.org/>), and the Human Genetic Variation Database (HGVD, <http://www.hgvd.genome.med.kyoto-u.ac.jp/>) to collate healthy controls in global and Japanese populations.

RESULTS

Patients and causative genes

Fifty-one patients from 36 families were clinically diagnosed with BOR syndrome, and causative genes were identified in 38 patients from 26 families. Thirty-four patients from 22 families were found to contain *EYAI* heterozygous mutations or partial deletions (Table 1); nonsense mutations

were identified in six families (SC7, SC19, SC100, SC347, SC381, and SC462), and frameshift mutations due to the deletion or duplication of several base pairs were identified in four families (SC170, SC219, SC242, and SC289). One patient (SC7) with a nonsense mutation and another with a frameshift mutation (SC289) have been already reported.^{8,9} The same missense mutation (R440Q) was detected in three different families (SC313, SC336, and SC434). Splice site mutations were identified in three families (SC46, SC251, and SC276); exonic deletions, identified by MLPA, were identified in six families (SC4, SC5, SC6, SC11, SC112, and SC316) (Table 1, Figure 1). One patient (SC101) had a *SIX1* heterozygous missense mutation. In two patients, the causative gene was identified as *SALL1*; one patient (SC26) found to contain a microdeletion at 16q12, which includes *SALL1*, has been already reported,⁵ and another patient (SC334) had a *SALL1* frameshift mutation caused by a single-nucleotide duplication (Table 2). Thirteen patients from 10 families with a BOR-like phenotype had no mutations in BOR-related genes (Supplementary Table 1).

Clinical phenotypes

The phenotypes and genotypes of the patients with *EYAI* aberrations are listed in Table 3. In addition, HL, which was found in all patients, PP, second branchial arch anomalies including CC or CF, and renal anomalies were highly common symptoms (> 60%).

Twenty-three patients with *EYAI* aberrations from 18 families were identified with renal

anomalies in this study. The most frequent type of renal disease in patients with *EYAI* aberrations was renal hypodysplasia (RHD), which was found in 10 patients (Figure 2). Unknown-cause renal dysfunctions were identified in seven patients. Over 50% of the patients with *EYAI* aberrations were diagnosed with RHD or unknown-cause renal insufficiency. Four patients (the mother of SC5 and SC100, SC170, and SC276) needed renal replacement therapy because of end-stage renal disease. One boy (the brother of SC19) suffered from pulmonary hypoplasia caused by bilateral renal hypoplasia, resulting in perinatal death. Even within the same families, the renal phenotypes often varied widely (Figure 3).

DISCUSSION

This study investigated several causative genes and clinical phenotypes relating to BOR syndrome in a Japanese population. We identified causative genes in 72.2% of patients in families clinically diagnosed with BOR syndrome. The detection rate in this study was higher than in our previous study.¹⁰ As we mainly analyzed typical BOR syndrome patients, this may have resulted in the high detection rate. *EYAI* was the most frequently identified gene in our study, consistent with previous reports on populations in Japan,¹¹ Taiwan,¹² and Western countries.¹⁰ We identified *EYAI* aberrations in 22 families. With the exception of one family (SC5 and her relatives), all *EYAI* mutations were located at the 3'-end of exon 6, which includes the eyes absent homologous region (EyaHR), a

highly conserved 271-amino acid sequence in the C-terminal region of the protein.¹³ This study did not identify any correlations between the genotype and phenotype of *EYA1* in BOR patients.

The following methods of genetic analysis were used for patients diagnosed with BOR syndrome: direct sequencing for 15 families, MLPA for six families, NGS for three families, and aCGH for two families. We subsequently performed MLPA analysis for undiagnosed patients with *EYA1* mutations through direct sequencing. We have previously reported another Japanese patient with a LINE1 insertion in *EYA1* who was diagnosed through MLPA.¹⁴ MLPA analysis is therefore useful for the diagnosis of BOR syndrome. We identified causative genes in three BOR patients (SC101, SC170, and SC334) by NGS. A boy (SC170) in which an *EYA1* aberration was identified through NGS using TS1 had severe renal insufficiency, HL, branchial anomaly, and mild ID. He also had a *SIX2* missense mutation (NM_016932.4: c.390C>G, p.Cys130Trp). The patient had no family history of BOR syndrome. The *SIX2* mutation was inherited from a healthy mother, but the *EYA1* variant was *de novo*. *SIX2* is an important protein for mammalian renal development, and previous reports have identified patients with renal hypoplasia and mutations in *SIX2*.^{15, 16} Xu *et al.* reported that Six2 interacts with Eya1 and Myc for regulation of nephron progenitor cells in mice.¹⁷ In patient SC170, the *SIX2* variant may have contributed to his severe renal disease, although his mother had no renal diseases. One patient (SC101) was identified with a *SIX1* aberration by NGS. This variant (NM_005982.3: c.519G>C, p.Lys173Asn) has not been reported in mutational or healthy control

databases. In general, BOR patients with *SIX1* mutations have a lower incidence of renal disease.³

This patient did not suffer from any renal diseases, consistent with previous reports.

SALL1 is a transcription factor that is essential for renal development.¹⁸ Two patients were identified with *SALL1* aberrations in this study. One male patient (SC334) with a *SALL1* frameshift mutation was compatible with the BOR phenotype, but not TBS; another patient (SC26) had a 16q12 microdeletion that included *SALL1*.⁵ Neither patient presented with preaxial polydactyly or imperforate anus, which are characteristic features of TBS. It is an extremely rare disease; the prevalence of TBS is estimated to be 1:250,000.¹⁹ However, Engels *et al.* reported one BOR patient with a *SALL1* frameshift mutation;⁴ therefore, it is essential to analyze *SALL1* in BOR patients without any *EYAI* or *SIX1* aberrations.

Renal anomalies are the most important complication for the prognosis of patients with BOR, because renal insufficiency may cause severe anemia, bone diseases, stunted growth, or cardiovascular disease. However, we did not identify a notable genotype-phenotype correlations for patients with BOR syndrome; even within the same family, it was impossible to predict any renal anomalies based on the presence or absence of *EYAI* mutations. One girl (SC100) needed renal replacement therapy, although her father, who had the same *EYAI* mutation, did not show any renal anomalies. One boy (the brother of SC19), who died 6 hours after birth, had a healthy mother with the same *EYAI* mutation. This unpredictability is important for the genetic counseling of families

with a history of BOR syndrome. The reason for these differences remains unknown, and further study is required to resolve this uncertainty.

One patient (SC211) with left renal agenesis, HL, and EEA without ID met the criteria to be diagnosed with BOR syndrome. He had no mutations in *EYA1*, *SIX1*, *SALL1*, or *SIX5*, but did have a karyotype abnormality (47, XY +mar). We performed aCGH and diagnosed him with chromosome 22 partial tetrasomy (also known as cat eye syndrome or Schmid-Fraccaro syndrome, #115470). Chromosome 22 partial tetrasomy has an autosomal dominant pattern of inheritance, but there was no family history of the syndrome in this case. Symptoms included EEA with conductive HL, renal agenesis, and eye abnormalities. He had bilateral ptosis without a coloboma. Intellectual disabilities from this syndrome were non-existent or mild, and the intelligence of the patient was normal. Therefore, if patients with symptoms of BOR syndrome have any eye abnormalities, chromosome 22 partial tetrasomy should be considered as a potential alternative cause, and chromosomal tests including a G-banding test or aCGH should be performed to rule this out.

No major *SIX5* mutations were identified in any of our study participants. Hoskins *et al.* reported that *SIX5* is a causative gene for BOR syndrome in 2007;²⁰ however, no other published reports have confirmed the association of *SIX5* with BOR syndrome, although a *SIX5* mutation has been reported to be the cause of CAKUT in two patients.^{15, 21} In a study on a Taiwanese population, no connection was found between BOR syndrome and *SIX5* mutations.¹¹ In addition, Krug *et al.*

found that a patient with BOR syndrome and a *SIX5* mutation also had a mutation in *EYAI*.⁹

Although further investigation is necessary, *SIX5* may not have a role in BOR syndrome, at least in East Asian populations. In this study, 13 patients from 10 families showed no mutations in BOR-related genes. NGS was performed on all patients, including TS1 or a comprehensive CAKUT gene analysis. Further studies using whole-exome or genome sequencing are necessary for the patients and their families. **From our results and latest findings, we suggest an algorithm for precise diagnosis in BOR syndrome (Figure 4).**

In conclusion, this was the first large-scale study to perform a genetic analysis on patients with BOR syndrome in a Japanese population. No obvious genotype-phenotype correlation was identified in this study. Renal abnormalities were especially unpredictable. We propose that MLPA analysis for *EYAI* and comprehensive NGS analysis will be useful for the detection of genes related to BOR syndrome.

Conflicts of Interest

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Figure Legends

Figure 1.

Locations of *EYAI* mutations identified in this study. EyaHR, eyes absent homologous region; ● nonsense, ◇ splice site, ▲ missense, ■ frameshift; solid line, exon deletion; broken line: undetermined region.

Figure 2.

Renal phenotypes in patients with *EYAI* mutations. ARR, abnormal renal rotation; CyK, cystic kidney; HN, hydronephrosis; MCDK, multicystic dysplastic kidney; ReA, renal aplasia; RHD, renal hypodysplasia; VUR, vesicoureteral reflux.

Figure 3.

Family trees showing different renal phenotypes within the same families. BOR, branchio-oto-renal syndrome.

Figure 4.

Flow chart for genetic diagnosis in BOR patients.

Table 1. Characteristics of BOR syndrome patients with *EYA1* abnormalities.

Family	Patient	Age	Sex	FH	Renal disease	HL, type	Other BOR symptoms	Other	Nucleotide change	AA change	Method	Ref. HGMD, ClinVar
1	SC4	9m	F		-	+	CF, PP	-				
	father	NA	M	+	-	+	CF, PP	-	rsa 8q13.3(EYA1exons10-18)x1		MLPA	
2	SC5	38	F		-	+	FNP, EEA, IEA, MEA	-				
	son	4	M	+	-	+	EACA, MEA, PP	-	rsa 8q13.3(EYA1exons2-3)x1		MLPA	
	mother	67	F		CKD¶	+, s	-	RA				
3	SC6	6	F	-	-	+	CF, EEA, IEA, PP	-	rsa 8q13.3(EYA1exons2-12)x1		MLPA	
4	SC7	21	F	-	r RHD	+, mi	CC, IEA, PP	IH, PCOS	c.880C>T	p.Arg294Ter	DS	CM980645, Ogawa ⁸
5	SC11	20's	F	-	r ReA, l HN	+	PP	-	rsa 8q13.3(EYA1exons12)x1		MLPA	
6	SC19	0	M		RHD	+	CC, CF, EEA, PP	-				
	mother	38	F		r RHD	+	CC, PP	-				
	brother	0†	M	+	r RHD, l ReA	NA	EEA	Pulmonary hypoplasia	c.1289G>A	p.Trp430Ter	DS	
7	SC46	6	M		l HN	+	CF, PP	-				
	brother	4	M	+	r HN	+	CF, PP	-	c.1140+1G>A		DS	CS137061
	father	NA	M		-	+	CF, EEA, PP	-	ND			
8	SC100	2m	F		CKD¶	+	CF, PP	UH				
	Father	NA	M	+	-	+	CF, PP	-	c.634C>T	p.Gln212Ter	DS	CM081585
9	SC112	1	F		-	+, mi	CF, FNP, IEA, PP	MDD, SS				
	mother	33	F	+	CKD	+	CF, PP	-	rsa 8q13.3(EYA1exons17)x1		MLPA	
	grandfather	60	M		CKD	+	CF, PP	RP				

	sister	5m	F	-	-	CC, PP	-						
10	SC170	6	M	-	CKD	+, c	CC, EEA, IEA, MEA, PP	mild ID	c.1643_1644dupAA	p.Val549LysfsTer7	NGS (TS)		
11	SC219	12	F	+	r RHD	+, c	CF, EEA, IEA, MEA, PP	hyperopia, SS	c.1122delA	p.Leu374PhefsTer6	DS	RCV000041385.2	
	mother	NA	F		-	+	CF, PP	-	ND				
12	SC242	2	M	+	-	+	EEA, FNP, PP	SS	c.1730_1745delACTC GGACCTCATGGC	p.His577ProfsTer57	DS		
	brother	NA	M		r RHD, HN	+	PP	-	ND				
	Father	NA	M		r RHD	+	EEA, PP	-	ND				
13	SC251	3	F	-	RHD, r CyK	+	PP	-	c.1050+2T>C		DS		
14	SC276	6	F	+	r RHD l MCDK	+, s	CF	-	c.418+1G>C		DS	RCV000315563.1*	
	mother	NA	F		-	+	NA	-	ND				
15	SC289	1	F	+	HN	+	CF, EACA, EEA, MEA, PT	Cavernous angioma	c.1161_1164delAGAT	p.Ile387MetfsTer12	DS		
	mother	NA	F		-	+	PP	-	ND				
16	SC313	1m	F	+	r ReA, l RHD	+	CC, EEA, FNP, PP	-					
	mother	28	F		CKD	+, c	CC, FNP, PP	-	c.1319G>A	p.Arg440Gln	DS	CM972838	
	brother	5	M		-	+	EEA, PP	-					
17	SC316	60	F	+	CKD	+	EEA	dysopia	rsa 8q13.3(EYA1exons17)x1		MLPA		
	daughter	NA	F		-	+	EEA	dysopia	ND				
18	SC336	9	M	-	r RHD	+	EEA	-	c.1319G>A	p.Arg440Gln	DS	CM972838	

VUR													
19	SC347	14	M	-	CKD	+, c	CC, EEA, FNP, hemifacial		-	c.922C>T	p.Arg308Ter	DS	CM970455
					urethrostenosis		microsomia						
20	SC381	25	M	+	r RHD	+	PP	-	c.880C>T	p.Arg294Ter	DS	CM980645	
	mother	NA	F		-	+	NA	ND					
21	SC434	16	F	+	r ARR	+, s	CF, PP	-	c.1319G>A	p.Arg440Gln	DS	CM972838	
			l RHD										
	grandfather	NA	M		-	+, s	CF, PP	-	ND				
	mother	36	F		-	+	PP	-	ND				
	sister	18	F	-	-	CF	-	ND					
22	SC462	1	F	-	-	+	CC, PP	PAS	c.698C>A	p.Ser233Ter	DS		

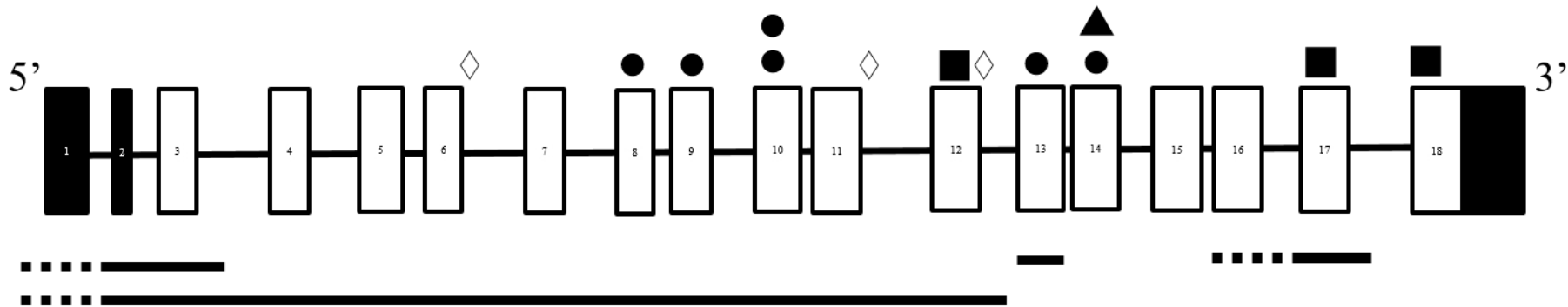
Abbreviations: ARR, abnormal renal rotation; BOR, branchio-oto-renal; c, conductive; CC, cervical cyst; CF, cervical fistula; CKD, chronic kidney disease (unknown-cause renal insufficiency); CyK, cystic kidney; DS, direct sequencing; EACA, external auditory canal anomalies; EEA, external ear anomaly, FNP, facial nerve palsy; HL, hearing loss; HN, hydronephrosis; HSK, horseshoe kidney; ID, intellectual disability; IEA, internal ear anomaly; IH, inguinal hernia; l, left; m, month; MCDK, multicystic dysplastic kidney; MDD, motor developmental delay; MEA, middle ear anomaly; mi, mixed; MLPA, multiplex ligation-dependent probe amplification; NA, not available; **ND, not done**; NGS, next generation sequencing; PAS, peripheral pulmonary artery stenosis; PCOS, polycystic ovary syndrome; PP, auricular or preauricular pits; PT, preauricular tag; PU, posterior urethral valve; r, right; RA, rheumatoid arthritis; ReA, renal aplasia; RHD, renal hypoplasia or dysplasia; RP, retinitis pigmentosa; s, sensitive; SS, short stature; TS1, TruSight One; UH, umbilical hernia.

†, fatal case; ¶, on renal replacement therapy.

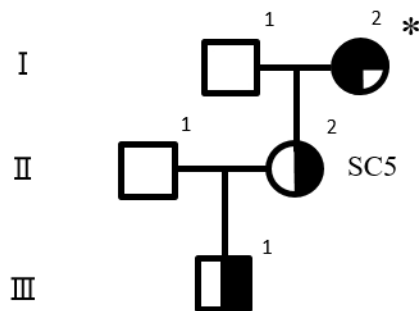
*RCV000315563.1 is registered as G>A

EYA1 NM_00503.4

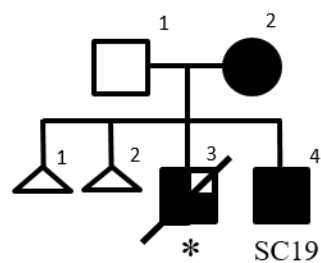
EyaHR



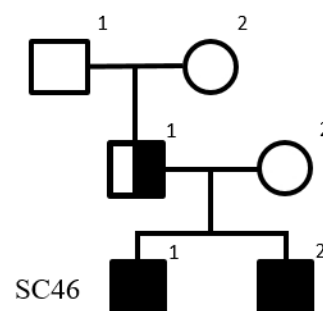
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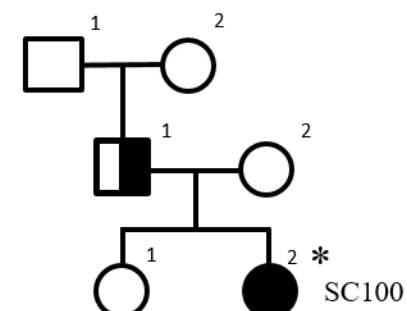
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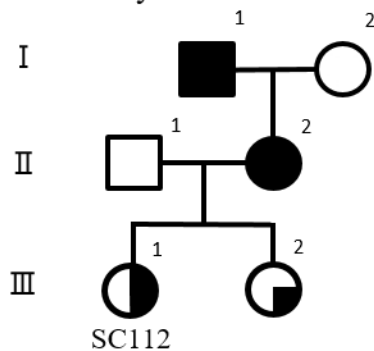
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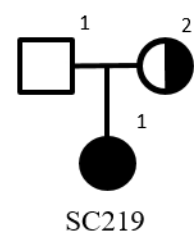
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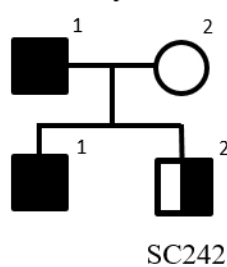
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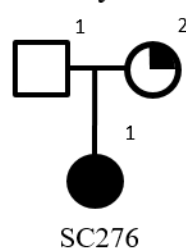
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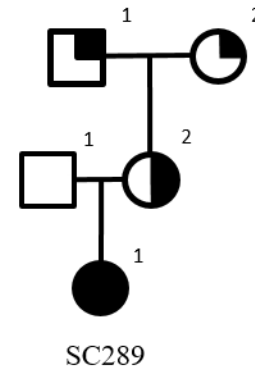
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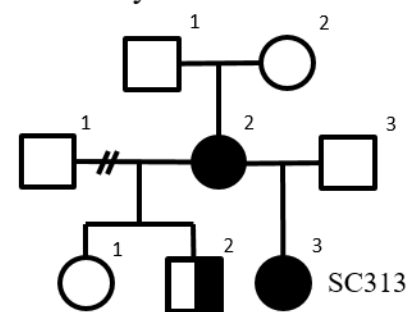
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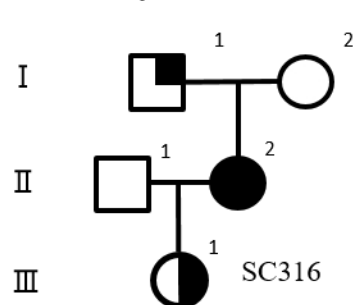
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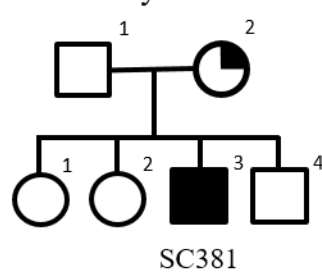
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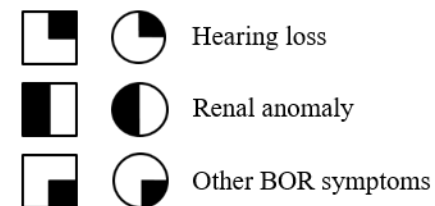
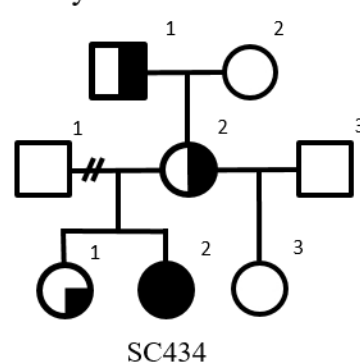
Family 17



Family 20



Family 21



* End-stage renal disease

