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Prenatal phenotypic delineation of a de novo *EYA1* likely pathogenic variant in branchio-oto-renal syndrome

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Abstract

Background Branchio-oto-renal (*BOR*; MIM 113650) syndrome is primarily linked to pathogenic variants in the *EYA1* gene. Although over 200 pathogenic variants of the *EYA1* gene have been reported, validation of the pathogenicity of novel variants and the aggregation of prenatal phenotypes are crucial to guide prenatal diagnosis.

Methods This study analyzed the clinical and genetic data of a fetus presenting with BOR syndrome. A de novo *EYA1* gene variant was identified and the functional impact of this variant was validated using minigene splicing assays in vitro. Additionally, a systematic review of prenatal cases with *EYA1* variants was conducted to summarize phenotypic frequencies.

Results Prenatal ultrasound detected left ear anomaly, facial cyst and a persistent right umbilical vein. Genetic testing revealed a novel variant c.640-15G > A in the *EYA1* gene. In vitro minigene assays demonstrated an aberrant effect on splicing. According to the American College of Medical Genetics (ACMG) guidelines, this variant was reclassified as likely pathogenic. Systematic literature review indicated that urinary system abnormalities and amniotic fluid anomalies were more prevalent in prenatal cases.

Conclusions This study adds a novel likely pathogenic variant to the *EYA1* variant spectrum in BOR syndrome and suggests that certain prenatal ultrasound phenotypic markers might be strongly associated with *EYA1*-related diseases.

Keywords EYA1 gene, Branchio-oto-renal syndrome, Whole-exome sequencing, Minigene splicing assay

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Introduction

Branchio-oto-renal (BOR; MIM 113650) syndrome is a rare autosomal dominant disorder and is clinically characterized by hearing loss, preauricular pits or tags, branchio fistulas or cysts, and renal abnormalities, with an incidence of 1/40,000, accounting for approximately 2% of profound deafness children [1, 2]. When renal dysplasia is not present, BOR syndrome is sometimes referred to as branchio-oto (BO) syndrome, which shares clinical symptoms and genetic factors similar to *BOR*. While diagnostic criteria proposed by Chang et al. are most widely used for the clinical diagnosis of BOR [3], it is usually not obvious or consistent enough to be recognized by routine prenatal ultrasound. In particular, hearing loss and preauricular pits are almost completely undetectable in prenatal ultrasound, making prenatal diagnosis of the disorder very difficult. Therefore, early identification of genetic variants underlying the pathogenic mechanism of BOR is of utmost importance.

Pathogenic variants in *EYA1*, *SIX1*, and *SIX5* have been identified as major genetic causes of BOR/BO syndrome [4]. Among them, *EYA1* is identified as the initial BOR/BO syndrome gene, accounting for approximately 40% of patients [5]. The *EYA1* gene, located on chromosome 8q13.3, encodes a transcriptional co-activator that interacts with *SIX* proteins to regulate organogenesis [6]. Hitherto, more than 200 reported pathogenic variants of the *EYA1* gene, including missense variants, nonsense variants, frameshift, and splice site variants, are associated with BOR/BO syndrome globally [7]. However, existing research has primarily focused on postnatal phenotypes, with limited data on prenatal phenotypes associated with *EYA1* variants in BOR/BO syndrome.

In this study, we found a de novo *EYA1* variant prenatally in a fetus presenting with ear anomalies. To investigate its pathogenicity and mechanism, we constructed the mutant and wild-type of *EYA1* variant by using minigene splicing assays to verify that its abnormal splicing during the translation process. Furthermore, we summarized reported prenatal cases of *EYA1*-associated disorders to evaluate the detectability of phenotypes via prenatal ultrasound. The finding of this study aims to add a novel likely pathogenic variant to the spectrum of the *EYA1* gene in BOR/BO syndrome and provide guidance for prenatal diagnosis and genetic counseling.

Methods

Clinical data

A pregnant woman underwent routine prenatal checkups in the Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Based on the prenatal ultrasound examination and genetic results, the father and mother requested termination of the pregnancy. We collected tissues from the induced abortion and peripheral blood from the parents of the fetus to investigate the cause of the fetal abnormalities.

DNA extraction

Genomic DNA was extracted from 200μL peripheral blood or 50 mg tissue samples, using a Qiagen DNA Blood Midi/Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Quality of genomic DNA was evaluated by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and agarose gel analysis.

Exome sequencing

50 ng genomic DNA was enzymatically fragmented to around 200 bp. The DNA fragments were then end repaired, and one A base was added to the 3' end. Secondly, the DNA fragments were ligated with barcoded sequencing adaptors, and fragments about 320 bp were collected by XP beads (Berry Genomics, China). After PCR amplification, the DNA fragments were hybridized and captured by Nano WES (Berry Genomics, China) according to the manufacturer's Protocol. The hybrid products were eluted and collected, and then subjected to PCR amplification and the purification. Next, the libraries were quantified by qPCR. Finally, Novaseq6000 platform (Illumina, San Diego, USA), with 150 bp pair-end sequencing mode, was used for sequencing the genomic DNA of the family. Raw image files were processed using CASAVA v1.82 for base calling and generating raw data. The sequencing reads were aligned to the human reference genome (hg38) using Burrows–Wheeler Aligner and PCR duplicates were removed by using Picard v1.57 (http://picard.sourceforge.net/). Verita Trekker® Variants Detection System by Berry Genomics and the third-party software GATK (https://software.broadinstitute.org/gatk/) were employed for variant calling. Variant annotation and interpretation were conducted by ANNOVAR and the Enliven® Variants Annotation Interpretation System authorized by Berry Genomics. Variants with minor allele frequencies (MAF) < 1% in exonic region or with splicing impact were taken for deep interpretation considering ACMG category, evidence of pathogenicity, and clinical synopsis and inheritance model of the associated disease. The variants were classified to five categories: pathogenic, likely pathogenic, uncertain significance, likely benign and benign, according to the American College of Medical Genetics and Genomics (ACMG) guidelines for interpretation of genetic variants.

Sanger sequencing

Sanger sequencing was carried out to confirm the mutations identified by exome sequencing. PCR fragments of target variants were amplified by primers (*EYA1*-F:CAGAACATTATCTTGACAGC,*EYA1*-R:ATGGACAGTCAGGTCAGGTAGGTG) under amplification conditions: 95℃ 3 min; 95℃ 30 s, 59℃ 30 s, 72℃ 20 s (35 cycle); 72℃ 4 min. PCR products were purified and sequenced using an ABI 3730xl DNA Analyzer with the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA).

Minigene splicing assay

The minigene assay was carried out to examine the cDNA region of target gene according to the candidate variant. Part of *EYA1* gene (Exon8(83 bp)- Intron8(404 bp)- Exon9(187 bp)- part of intron9(498 bp)) were split and constructed in pcMINI-N plasmids. Part of *EYA1* gene (Exon8(83 bp)- Intron8(404 bp)- Exon9(187 bp)- part of intron9(864 bp)- Exon10(140 bp)) were split and constructed in pcDNA 3.1 plasmids.The wild and mutant plasmids were confirmed by Sanger sequencing, then plasmids were transfected into HeLa and HEK293T cells separately using classical restriction and ligation methods. Total RNA was extracted from cells cultured for 48 h with Trizol (RNAiso PLUS) (TaKaRa, Kusatsu, Japan) and reverse-transcribed with the Superscript III reverse transcriptase (HifairTM 1 st Strand cDNA Synthesis SuperMix for qPCR (YEASEN, Shanghai, China)), and the resulting cDNA was PCR-amplified. The amplified products were analyzed on 1% agarose gel electrophoresis. Compare the sequencing results of wild and mutant-type in the cells and analysis the splice form of the mutant type. To substantiate the conclusion drawn in this study, the assay was conducted repeatedly in pcMINI-N and pcDNA 3.1 plasmids (Table 1) by using the same experimental method as above.

Table 1 Primer sequences used in minigene assays

Primer name	Primer sequence (5′- 3′)
EYA1-mut-F	AGACAATTCTTGTGTAGTTTGATTTGGTAGG
EYA1-mut-R	CCTACCAAATCAAACTACACAAGAATTGTCT
pcDNA3.1-joint-F	AAAAGTGAGCCTCTGTCACCTCTTTGCATG
pcDNA3.1-joint-R	CATGCAAAGAGGTGACAGAGGCTACATTTT
EYA1-pcDNA3.1-XhoI-R	TAGACTCGAGCTCAAGATCAGAATCTGGGG
EYA1-pcMINI-N-KpnI-F	GCTTGGTACCATGGGTAGCAGTTTTACAACATCA
EYA1-pcMINI-N-XhoI-R	TTTCCTCGAGTGCCCAAACTCATGACACAT
pcDNA3.1-F	CTAGAGAACCCACTGCTTAC
pcDNA3.1-R	TAGACTCGAGCTCAAGATCAGAATCTGGGG
pcMINI-N-F	GCTTGGTACCATGGGTAGCAGTTTTACAACATCA
pcMINI-N-R	TAGAAGGCACAGTCGAGG

Literature search using PubMed

We conducted a systematic literature search, with reference to Tian et al [8], to identify studies published up to July 1, 2025, that focused on case reports related to the EYA1 gene and BOR syndrome. Subsequently, we selected literature that included prenatal ultrasound or phenotypic descriptions and summarized them.

Ethical approval

Written informed consent was obtained from the parents of the fetus for the whole exome sequencing and in vitro experiment. The study protocols were approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Ethical Approval Number: UHCT250465). This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. This study informed the family and obtained their consent for publication of clinical details and fetal images from parents.

Results

Prenatal ultrasound and genetic testing results

As shown in Fig. 1A, prenatal ultrasound revealed a persistent right umbilical vein (PRUV) and a facial cyst measuring 0.6 cm × 0.4 cm × 0.6 cm located below the left oral commissure, suggestive of a branchial cleft cyst in the fetus at 26-week gestation. The fetal right ear measured 2.1 × 1.5 cm, while left ear was smaller (1.4 × 0.7 cm) with abnormal morphology. The results showed that a de novo variant c.640-15G > A in the *EYA1* gene was identified through exome sequencing in Fig. 1B. According to the ACMG guidelines, this variant was classified as a variant of uncertain significance (VUS) based on the following evidence: PP3: this variation may affect splicing by the prediction of spliceAI and MaxEntScan. The prediction indicated that the c.640-15G > A variant might create a novel cryptic splice acceptor site with a maximum delta score of 0.87 (a delta score ≥ 0.5 indicates a high probability of splice disruption), suggesting a strong potential for aberrant splicing induced by this variant (Supplementary Fig. 1 F); PM2_Supporting: this variant is absent in reference population databases, including the 1000 Genomes Project, the Exome Aggregation Consortium and the Genome Aggregation Database. Postmortem fetal morphological assessment was consistent with prenatal ultrasound results (Fig. 1C).

Minigene analysis

To further investigate the potential impact of this variant on splicing, a minigene assay was conducted. As shown in Fig. 2, the variant c.640-15G > A disrupted normal mRNA splicing. The results from both the pcDNA3.1 and pcMINI-N vectors were

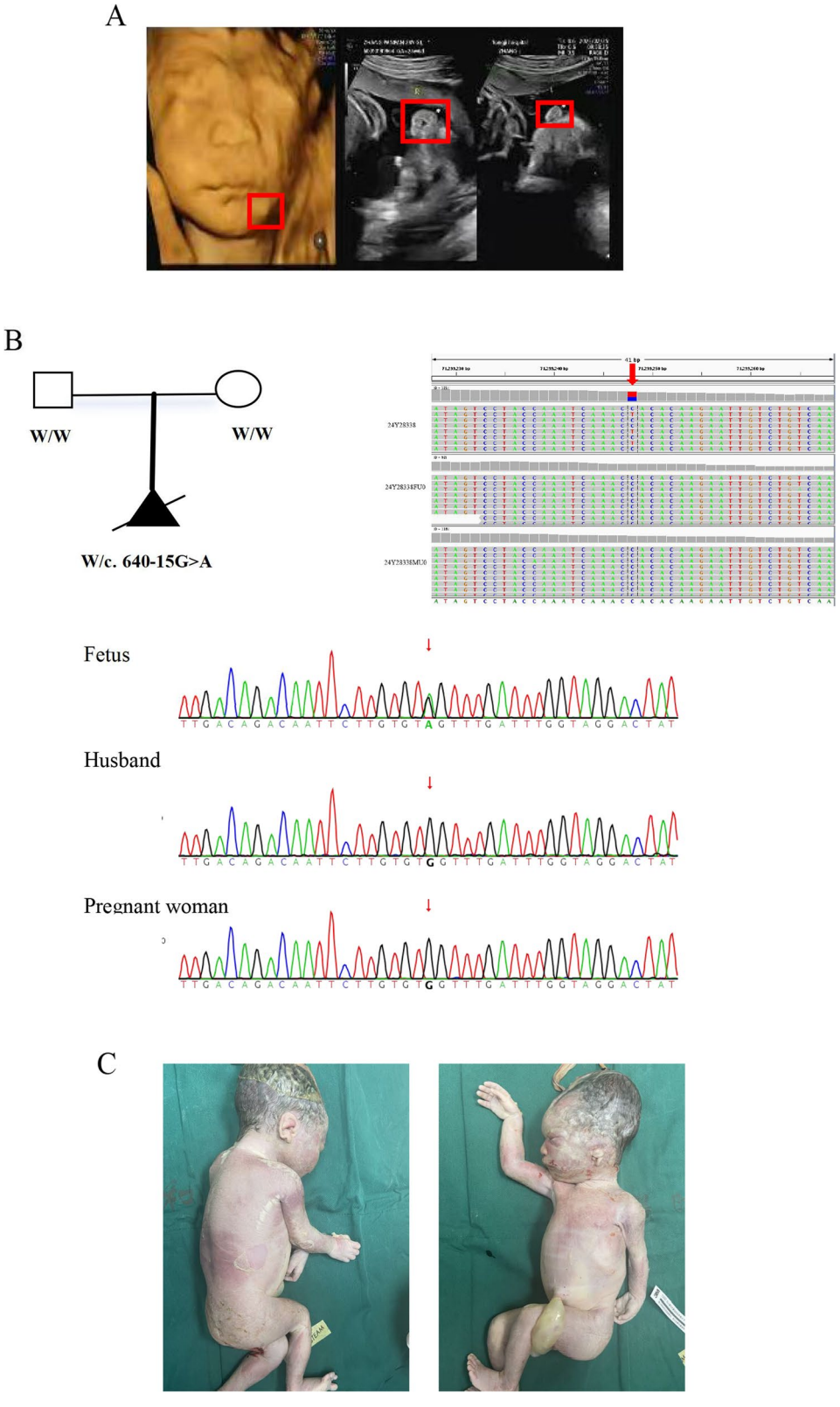

Fig. 1 Prenatal ultrasound findings, genetic analysis, and postmortem photograph of the fetus with a de novo EYA1 variant. **A** Abnormal prenatal ultrasound of the fetus. **B** Familial Sanger sequencing results and exome sequencing for EYA1 gene (NM_000503.6) c.640-15G > Avariant. **C** The picture of postmortem fetal

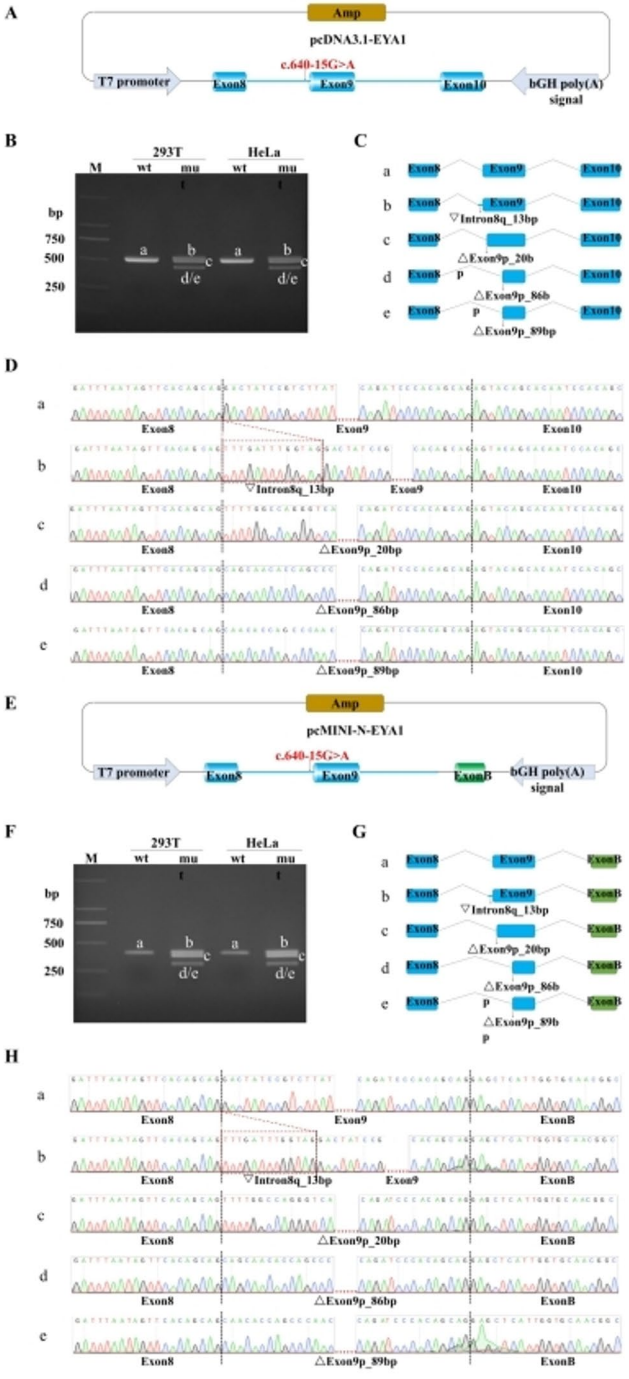

Fig. 2 Minigene detection of the EYA1 gene variant c.640-15G > A. **A**,**E** Schematic diagram of minigene plasmid construction. Exons 8, 9, and 10 were cloned into the pcDNA3.1 and pcMINI-N vectors. **B**,**F** Agarose gel electrophoresis of RT-PCR products from 293 T and HeLa cells transfected with wt and mut minigene constructs. (a) Wild-type splicing product; (b, c, d, e) Aberrant splicing products of mutant constructs. **C**,**G** Schematic diagram of splicing patterns. (a) Correct splicing observed in the wild type; (b, c, d, e) Aberrant splicing products caused by the c.640-15G > A mutation. **D**,**H** Sequencing results corresponding to the different splicing bands (a) Correct splicing observed in the wild type; (b, c, d, e) Abnormal splicing products caused by the c.640-15G > A mutation

consistent, with the variant leading to four abnormal splicing scenarios: 1) A 13-bp retention at the 3' end of Intron 8, resulting in a 234aa truncated protein (p.Asp214PhefsTer22); 2) A 20-bp deletion at the 5' end of Exon 9, resulting in a 223aa truncated protein (p.Asp214PhefsTer11); 3) An 86-bp deletion at the 5' end of Exon 9, resulting in a 258aa truncated protein (p.Asp214GlnfsTer46); 4) An 89-bp deletion at the 5' end of Exon 9, resulting in a 257aa truncated protein (p.Asp214GlnfsTer45). Therefore, based on ACMG guidelines, this variant is reclassified as likely pathogenic (PVS1 (RNA), PS2_Supporting and PM2_Supporting) [9].

Systematic characterization of prenatal phenotypes in EYA1-related disorders

To delineate the prenatal phenotypic spectrum of EYA1-related disorders, we conducted a secondary systematic analysis of prenatal cases with EYA1 pathogenic variants, based on the well-characterized cohort of 17 cases reported by Tian et al [8, 10–23]. Among the reported prenatal cases, urinary system anomalies were the most prevalent, observed in 6 of 17 cases (35.3%). The specific manifestations within this category comprised bilateral renal pelvic dilation, small kidneys, bilateral renal agenesis, and bilateral hydronephrosis, consolidating the pivotal role of EYA1 in renal development and establishing urinary tract abnormalities as a primary prenatal indicator.Amniotic fluid abnormalities and heart abnormalities were each present in 4 cases (23.5%). Notably, the spectrum of heart defects included echogenic intracardiac focus, interrupted aortic arch, ventricular septal defect, and tetralogy of Fallot. Ear anomalies were less frequently detected prenatally, identified in only 2 cases (11.8%). Additionally, lung hypoplasia, limb anomalies, facial cyst, and umbilical cord abnormalities were each reported in a single case (5.9% each). In the present study, the proband was found to have left ear anomaly, facial cyst (highly suspected to be a branchial cleft cyst), and PRUV by prenatal ultrasound. The ear anomaly was consistent with the reported core phenotype in prenatal cases, while PRUV has not been specifically documented in prenatal cases of *EYA1*-related disorders to date.

Discussion

The considerable clinical heterogeneity of BOR/BO syndrome, particularly manifested by atypical presentations such as mild structural abnormalities of ear with hearing loss, preauricular pits, and no accompanying renal abnormalities, makes it difficult to detect via routine prenatal ultrasound screening, posing a significant challenge for prenatal diagnosis. Thus,

combining suspicious prenatal ultrasound findings with genetic testing is critical to make prenatal diagnosis of BOR/BO syndrome. Given the limitation of reported prenatal *EYA1*-associated phenotypes, our study identified a novel variant (c.640-15G > A) in the *EYA1* gene through exome sequencing and validated its effect by minigene assays.

EYA1, characterized by a divergent N-terminal activation domain and a conserved C-terminal *EYA* domain, is a major pathogenic gene of BOR syndrome and plays a crucial role in the development of the ear, kidney and branchial arches [24]. Mechanistically, *EYA1* functions as a co-activator by directly binding *SIX1* to form a bipartite transcription complex. This interaction constitutes the molecular basis for BOR/BO syndrome pathogenesis, wherein disruption impairs transcriptional activation of downstream targets essential for branchial arches, otic, and renal system development [9]. Furthermore, haploinsufficiency from reduced *EYA1* dosage also constitutes a primary pathogenic mechanism in BOR/BO syndrome [25].

In our case, the proband was initially detected with left ear anomaly, PRUV, and facial cyst by prenatal ultrasound. Subsequently, a de novo intronic variant with uncertain clinical significance was initially detected in the fetus via exome sequencing and was absent in both parents. Although no abnormalities in the urinary system were detected by prenatal ultrasound and it was also not clear whether the fetus would have any anomalies in hearing or branchial arches after birth, the combination of ear anomaly with a de novo variant in *EYA1* gene raised significant concern for severe BOR/BO syndromic involvement. After multidisciplinary counseling on the potential phenotypic spectrum and prognostic uncertainty, the family opted for pregnancy termination. As shown in Fig. 1, the fetus presented with left ear anomaly, facial cyst (suspected branchial cleft cyst) and PRUV, which was consistent with ultrasound findings. However, comprehensive evaluation of the renal system and internal otic structures was not performed. To definitively establish the pathogenicity of the novel variant, we constructed pcMINI-N and pcDNA 3.1 plasmids in minigene assays to validate the accuracy of the results. The results showed that c.640-15G > A caused the retention of intron 8 and deletion of exon 9, which leads to reading frame shifts and the generation of premature termination codons, thereby affecting the splicing of the gene. Thus, we deduced that the resulting abnormal transcript may trigger nonsense-mediated mRNA decay (NMD) and be degraded by NMD, ultimately leading to *EYA1* haploinsufficiency. The pathogenic effect of c.640-15G > A on splicing was first indicated by in silico prediction and this predictive result was highly consistent with the findings of our in vitro minigene splicing assay, which confirmed that the variant indeed induced aberrant splicing events including intron 8 retention and partial deletion of exon 9. As a widely used in silico tool for splice site variant prediction, SpliceAI provides a reliable preliminary screening basis for identifying potential pathogenic intronic variants. However, it is worth noting that in silico prediction alone cannot be used to confirm the pathogenicity of splice variants, as the prediction results are only based on sequence characteristics and lack functional evidence of actual splicing disruption. This is also the reason why we further performed minigene splicing assays to verify the predictive results. The same effect has been reported in c.867 + 5G > A and c.639 + 3A > C, in which both variants were found to produce a truncated transcript of *EYA1* lacking exon 8 [26]. Based on ACMG guidelines, c.640-15G > A is reclassified as likely pathogenic.

Furthermore, we summarized reported prenatal phenotypes associated with novel *EYA1* variants, including renal, skeletal, cardiac, pulmonary, and otic anomalies. This multi-system involvement suggests that different variants of the *EYA1* gene may drive divergent pathogenic mechanisms. The high prevalence of urinary system anomalies in prenatal cases establishes renal malformations as the most critical prenatal ultrasound indicator for BOR syndrome. Notably, atypical ultrasound findings such as bilateral caliectasis and renal pelvis dilation may resolve during gestation, potentially evading detection and contributing to underdiagnosis. Amniotic fluid volume, serving as a critical protective buffer for fetal development, demonstrates clinical relevance in prenatal diagnosis of *EYA1*-associated disorders. Specifically, oligohydramnios was identified in 17.6% of cases in prenatal phenotypes and this association may be mechanistically explained by urinary system malformations, aligning with the role of *EYA1* in nephrogenesis and the renal phenotype of BOR syndrome. Congenital heart defects, observed in 4 cases, represented a rare but potential prenatal phenotype in *EYA1*-related disorders, warranting expanded investigation into their pathogenic association. Other prenatal ultrasound phenotypes,including polyhydramnios, lung hypoplasia, limb anomalies, facial cyst and PRUV, are infrequent and may represent incidental developmental variants, yet their co-occurrence with *EYA1* defects demands attention. Finally, the absence of discernible prenatal ultrasound abnormalities in 30% of cases may result from technical limitations of routine prenatal ultrasound and age-dependent penetrance where certain manifestations emerge postnatally. Compared with previously reported prenatal cases of *EYA1*-related disorders, the present case displays both overlapping and distinct features. Consistent with the characterized phenotype of *EYA1*-related disorders, ear anomalies and facial cyst were observed, reinforcing their

association with *EYA1* dysfunction. However, the presence of PRUV in our case has not been previously documented in association with *EYA1* variants, warranting further investigation.

Although the novel intronic *EYA1* variant (c.640-15G > A) was reclassified from uncertain significance to likely pathogenic based on correlative clinical features and functional minigene assays, this study has limitations: 1) direct detection of the aberrant transcript in vivo remains lacking, 2) the impact of aberrant splicing on *EYA1* function is not clear, and RNA-sequencing was not applied in the proband to validate the precise splicing effects, 3) the type of facial cyst remains unclear, and its co-occurrence with PRUV in the context of *EYA1* defects could be incidental, 4) the limited sample precludes penetrance estimation and hinders comprehensive exploration of genotype–phenotype correlations in BOR/BO syndrome, 5) the possibility of parental gonadal mosaicism is not ruled out and potentially leads to an underestimation of recurrence likelihood. Future studies should elucidate the molecular pathology of aberrant splicing at c.640-15G > A and establish corresponding knock-in models to analyze specific tissue phenotypes particularly in branchial arches, hearing and renal systems, thereby expanding the clinical implications of this finding.

In conclusion, a de novo variant (c.640-15G > A) of *EYA1* gene was discovered in the fetus and defined as a pathogenic variant through integrated prenatal ultrasound and functional minigene assays. Our systematic aggregation of prenatal phenotypes linked to pathogenic *EYA1* variants reveals a wide range of phenotypic spectrum in BOR/BO syndrome, underscoring the necessity of combining prenatal ultrasound with genetic testing. The findings of our study not only broaden the variant spectrum of the *EYA1* gene but also provide valuable prenatal diagnosis and genetic counseling for affected families.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12920-026-02366-x.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

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Authors' contributions

Yin Zhao and Yang Zhang designed the study. Lei Sun and Defeng Shu performed the research, analysed the data and wrote the paper. Wencong He, Ruilin Ma, Hui Tao, Zejun Yang, Yanan Li, Ziyang Liu collected the clinical samples and participated in the discussions. All authors have reviewed and approved the manuscript.

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Data availability

The *EYA1* variant found in this study was submitted to the Clinvar repository (https://submit.ncbi.nlm.nih.gov/subs/variation_clinvar/SUB15633396/).

Declarations

Ethics approval and consent to participate

The study protocols were approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Ethical Approval Number: UHCT250465). This study was performed in strict compliance with the principles of the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

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