A novel mutation of the EYA1 gene in a branchio-otic syndrome child with secretory otitis media and bilateral vestibular weakness

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Research Article

Keywords: Branchio-otic syndrome, EYA1 gene mutation, whole exome sequencing, secretory otitis media, bilateral vestibular weakness

Posted Date: February 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2553494/v1

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Abstract

Objective

To investigate the phenotypic manifestations and molecular etiology of branchio-otic syndrome (BOS) in a Chinese family.

Methods

We recruited two generations of a Chinese family with BOS. Family history was obtained and detailed physical and hearing examinations were performed on all family members. Whole-exome sequencing (WES) was used to screen the candidate disease genes using phenolyzer software. Sanger sequencing was used for validation. The pathogenicity of the candidate mutations was analyzed.

Results

The proband had severe-to-profound sensorineural hearing loss in the left ear, and mixed hearing loss, type III cup-shaped ear, external auditory canal stenosis and cochlea hypoplasia in the right ear; Cochlear hypoplasia together with the fused lateral semicircular canal and vestibular in the left ear, with bilateral preauricular pits and branchial fistulae. Moreover, the patient had unilateral secretory otitis media (SOM) in the right ear and bilateral vestibular weakness (BVW), which has not been reported in previous studies. The patient’s hearing on the right side was restored to nearly normal after myringotomy with grommet insertion. We also identified a novel frameshift mutation in the proband (c.1697_1698delinsT[p.Lys566Ilefs*73]) in exon 17 of the EYA1 gene, which was assessed as “pathogenic” according to American College of Medical Genetics and Genomics guidelines. Sanger sequencing was used to validate the novel heterozygous mutation and WES accuracy.

Conclusion

This is the first report of a child with BOS with SOM and BVW, further enriching the known phenotypes of this gene mutation. We also observed a novel EYA1 gene mutation site in a patient with BOS, expanding the mutation map and providing a reference for genetic diagnosis.

1. Introduction

Branchio-oto-renal spectrum disorder (BORSD) is an autosomal dominant disorder characterized by branchiogenic malformations, varying degrees of hearing loss, and renal involvement. BORSD consists of branchio-otic syndrome (BOS) and branchio-oto-renal syndrome (BORS), with renal involvement as the distinguishing characteristic. The incidence of this disorder is approximately 1:40,000, accounting for
approximately 2% of children with severe deafness. Molecular confirmation has been performed in patients with BORS, which revealed that the most known causative genes were EYA1, SIX1, and SIX5. Of these genes, EYA1 accounted for the majority of diagnosed patients. Genetic mechanisms, such as frameshifts and genomic rearrangements, occur in this disorder. However, no unique relationship has been discussed between the nature of the mutations and the variable clinical features associated with BORSD. Whole-exome sequencing (WES) is an efficient strategy for selecting the coding sequences of a genome, covering 85% of pathogenic mutations, and has shown significant advantages in genetic diagnosis.

Herein, we report the case of an 8-year-old child from a Chinese family with BOS related to pathogenic variation in the EYA1 gene. A detailed assessment of the family history, physical examinations, and imaging evaluations were performed on the proband and other family members. In addition to typical clinical manifestations, the child also had secretory otitis media (SOM) which had not been previously reported in other studies. Furthermore, we performed WES and Sanger sequencing to analyze the genotypes and phenotypes of BORSD and then analyzed the pathogenicity of the candidate mutation. We conducted this study to expand both the mutational and phenotypic spectra of BOS and to analyze the key mutational site as an etiologic factor for such disorders.

2. Materials And Methods

2.1 Individual and clinical examinations

The two generations from one family recruited in our study comprised four people; the proband, his parents, and his brother (Fig. 1). Their clinical histories were obtained, and they underwent detailed physical examinations. The proband was an 8-year-old boy diagnosed with BOS who presented at our hospital with bilateral hearing loss, preauricular pits, and branchial fistulae, along with an external ear anomaly in the right ear (Fig. 2A) and inner ear anomalies (Fig. 3). Moreover, he had SOM in the right ear (Fig. 3D), which caused further mixed hearing loss and ear fullness. He subsequently underwent myringotomy with grommet insertion at our hospital's Otolaryngology Department. Micro-otoscopy and pure-tone audiometry (PTA) were used for audiological assessment to evaluate hearing levels (Fig. 2B). High-resolution computed tomography (HRCT) of the temporal bone was performed to determine the middle and inner ear morphologies (Fig. 3). Renal examinations, including renal ultrasonography, renal function tests, and analyses, were performed to screen for renal abnormalities. Three months after surgery, objective audiometry comprised of acoustic immittance, distortion product otoacoustic emission, auditory brainstem response (ABR), and auditory steady-state response were conducted. Vestibular function and vestibular evoked myogenic potential (VEMP) were also assessed. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Second Xiangya Hospital (certificate number: k074).

2.2 Genomic DNA extraction and quality assessment
Peripheral blood samples were collected from the proband and his family members. Genomic DNA was extracted using a DNA extraction kit (Agilent Technologies, Santa Clara, CA, USA), and the DNA quality of the samples was controlled using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) ultramicro nucleic acid protein analyzer. Purified DNA was quantified using Qubit (Thermo Fisher Scientific).

2.3 Library construction, capture, and WES

The SureSelect Human All ExonV6 capture system (Agilent Technologies) was used for library construction and capture. The latest optimized experimental procedures were strictly followed. First, the Covaris fragmentation instrument was used for genomic DNA fragments with a length of 100–500 bp, which were randomly interrupted. Next, a DNA library with a specific index was prepared by pooling the ends of the fragments after repair. The tail was then added, after which liquid-phase hybridization was completed with the biotin-labeled probe. Then, streptomycin magnetic beads were used to capture the exons of the genes. Next, polymerase chain reaction (PCR) linear amplification was used for library construction, and the library was analyzed using an Agilent 2100 bioanalyzer instrument (Agilent Technologies). Finally, Fastq data were obtained from an Illumina HiSeq 2500 sequencer by high-throughput sequencing using the 2 × 150 bp double-terminal sequencing mode.

2.4 Data screening and bioinformatics analysis

After quality control, the Fastq data were aligned to hg38 by Burroughs-Wheeler Aligner 6, and GATK software (Broad Institute of MIT and Harvard, Cambridge, MA, USA) was used to analyze Single Nucleotide Variant (SNV) small fragment insertion/deletion (InDel). Data filtering was completed using the dbSNP, ExAC03, HapMap, and 1000 Genomes databases, and sites with a mutation frequency of less than 1% were retained. Annotation analysis, site screening, and priority classification were performed for detected SNV/InDel, and the site with the highest priority was defined as “First1.” Meanwhile, for all the above SNV/InDel sites, the sequencing depth of each site in diseased samples and standard samples was considered, and the code written in Perl was used for deep filtering (the result < 5X was removed) to obtain the detailed mutation information of all samples. Finally, to predict gene pathogenicity, we used phenolyzer software (http://phenolyzer.wglab.org/) for the candidate gene study of BOS according to the genetic pattern analysis of the gene in the preliminary filter, retaining the “First1” gene as the candidate gene.

2.5 Sanger sequencing

Candidate variants were validated using Sanger sequencing to verify the DNA sequence variants detected by WES. Primers were designed for the target region using Primer 3 online software (http://bioinfo.ut.ee/primer3-0.4.0/). The forward primers were F: 5-CTGCACATATTATCAGCTACGTTCACA-3; the reverse primers were R: 5- CACTAGAAAAGAAAGCTGTGGAG-3. After purification with shrimp alkaline enzyme and exonuclease I (Agilent Technologies), the PCR products were sequenced on an ABI3730XL Genetic Analyzer. The sequence reads were analyzed using the Polyphred software (http://phred.org).
3. Results

3.1 Clinical manifestations and family member characteristics

Family history investigation and examinations revealed that only the child showed BOS (preauricular pits, branchial fistulae, type III cup-shaped ear, external auditory canal stenosis, hearing loss, and inner ear anomaly) clinical manifestations (Fig. 2A), whereas the other family members neither presented with any such clinical manifestations, nor had a history of ototoxic drug or noise exposure. The preoperative auxiliary examination results were as follows: Pure-tone thresholds showed severe-to-profound sensorineural hearing loss in the left ear, and mixed hearing loss, in which the average air conduction threshold was 59 dB, and the bone conduction threshold was 26 dB in the right ear (Fig. 2B). Acoustic immittance tests showed type “A” tympanogram in the left ear and type “B” tympanogram in the right ear that indicated SOM (Fig. 2C). Acoustic reflex could not be obtained in both ear through 500 to 4000 Hz. ABR for air conduction in both ears failed to elicit a response at 97 dBnHL. The videonystagmography caloric test revealed bilateral hyporeflexia with reflexivity of 5.0/s and 2.6/s on the left and right sides, respectively, after stimulation with cold water and 4.5/s and 5.5/s on the left and right sides, respectively, after stimulation with hot water. VEMP were absent after stimulation on both sides. HRCT of the temporal bone indicated soft tissue shadow in the right middle ear. Moreover, HRCT indicated cochlear hypoplasia in the right ear. Cochlear hypoplasia and the lateral semicircular canal fused with the vestibular into a single cavity in the left ear (Fig. 3). Abdominal color ultrasound and routine urine and blood biochemical examinations showed no abnormalities. The proband underwent myringotomy with grommet insertion in the right ear with visible viscous uid accumulation in the middle ear. Consequently, the postoperative ABR was 30 dBnHL in the right ear, indicating significant improvement as compared with the preoperative value.

3.2 Genetic and molecular analysis

3.2.1 Quality control of WES

After quality assessment of the raw data and comparative analysis with reference sequences, we obtained the following quality assessment data: on average, we generated > 12 G of total data per sample. The average ratio of the base Q30 was > 91%. The average mean coverage sequencing depth for the official target was 106×. When measured at a depth of 20×, 97% of the target was covered. These results suggest that the data captured by WES is adequate for reliably detecting DNA variants for further analysis.

3.2.2 Candidate gene screening

For annotation analysis of SNV/InDel, we selected two typical results. First, SNV/InDel sites were classified according to their position relative to the genome mutation (Fig. 4A) and functional positions (Fig. 4B). Based on the software analysis, we obtained the genetic correlation between the BOS and
priority score. Finally, the 10 candidate genes with the highest scores were screened. The histogram of the top 10 gene scores is shown in Fig. 4C, and the EYA1 gene had the highest score.

3.2.3 Discovery and validation of mutation sites

Further bioinformatic analysis was conducted on the WES results. After comparison with the database, a heterozygous mutation of c. 1697_1698delinsT (p. Lys566Ilefs *73) was found in exon 17 of the EYA1 gene in the proband (II:1), which caused the 566th amino acid of the coding protein to change from lysine to isoleucine. Mutational loci were not observed in dbSNP, ExAC, ESP6500, or 1000 Genomes databases and did not exist in the average population. In our study, the parents and brother of the proband were not detected the EYA1 gene mutation at this site. To verify the WES results, we performed Sanger sequencing of the family and observed that the proband had the EYA1 gene c. 1697_1698delinsT mutation (Fig. 4D), whereas the healthy family members had no mutation at this site.

4. Discussion

BOS is a rare autosomal dominant disorder with high genotype heterogeneity. It is differentially expressed in different individuals with incomplete dominance, and there is currently no evidence of a correlation between genotype and phenotype. Chang et al. proposed widely accepted diagnostic criteria for BORS using the major and minor criteria in 2004 (Table 1). The major criteria included a second branchial anomaly, hearing loss, preauricular pits, and renal anomalies. The minor criteria included external, middle and, inner ear anomalies, preauricular tags, facial asymmetry, and palatal abnormalities. BORS is clinically heterogeneous, with highly variable penetrance, even within the same family. Patients without a family history were diagnosed with BORS if they met three or more of the above major criteria or two major and at least two minor criteria. Chang et al. reported the frequency of the main features of BORS as follows: hearing impairment, 98.5%; preauricular sinus, 87.0%; malformed auricles, 86.8%; second branchial arch fistula/cyst, 86.5%; and renal anomalies, 58.3%. From a clinical standpoint, hearing loss is the most common and constant feature of EYA1 mutations and is detected in more than 90% of affected individuals. BOS treatment is mainly symptomatic, including the surgical removal of fistulae and cochlear implants. Prenatal testing may be an important means to prevent this disease. However, this requires further exploration.
Table 1
Clinical Criteria for BORS†

<table>
<thead>
<tr>
<th>Level</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major criteria</td>
<td>Branchial anomalies</td>
</tr>
<tr>
<td></td>
<td>Hearing loss</td>
</tr>
<tr>
<td></td>
<td>Preauricular pits</td>
</tr>
<tr>
<td></td>
<td>Renal anomaly</td>
</tr>
<tr>
<td>Minor criteria</td>
<td>External ear anomaly</td>
</tr>
<tr>
<td></td>
<td>Middle ear anomaly</td>
</tr>
<tr>
<td></td>
<td>Inner ear anomaly</td>
</tr>
<tr>
<td></td>
<td>Preauricular tags</td>
</tr>
<tr>
<td></td>
<td>Facial asymmetry</td>
</tr>
<tr>
<td></td>
<td>Palate abnormality</td>
</tr>
</tbody>
</table>

BORS, branchio-oto-renal syndrome

†Affected individuals must meet at least three major criteria, two major criteria and at least two minor criteria, or one major criterion for BORS and an affected first-degree relative (Chang et al. [2014]).

✓: Clinical symptoms of the proband.

In this case, the proband had bilateral preauricular pits, bilateral cervical branchial cleft fistulae, hearing loss, an external ear anomaly in the right ear, and bilateral inner ear anomalies. In addition to the above symptoms, the patient had SOM and BVW, which was not previously reported in other studies. As we all know, the SOM in an 8-year-old child is not rare. However, in our study, the occurrence of SOM is exactly the same side as external ear anomaly. This phenomenon can greatly indicate that BOS may affect the ventilation of the ear, like Eustachian tube, which further resulting in SOM. The most common etiology of bilateral vestibular hyporeflexia is gentamicin ototoxicity, followed by many rarer entities such as autoimmune inner ear disease, meningitis, bilateral Ménière’s disease, bilateral vestibular neuritis, and bilateral vestibular schwannomas. Regarding the abnormal vestibular function results in this study, we conducted the related test after surgery. Surprisingly, we found this child had bilateral vestibular hyporeflexia. Furthermore, HRCT (Fig. 3E) indicated that lateral semicircular canal malformation together with the vestibular fused into a single cavity in the left ear. This maybe the potential cause why BVW occurred. Moreover, it can inspire us to pay more attention to the correlation between vestibular function and BOS in the future.

WES can now sensitively detect sequence variants. In this context, we clarified the SNV/InDel using read-depth analysis of WES data in a family with BOS and concluded that 10 genes were most closely related
to BOS by pathogenic gene prediction, with $EYA1$ having the highest score. In addition, we identified a novel $EYA1$ mutation in the family, which was further verified by Sanger sequencing as a $de$ $novo$ frameshift mutation. The $EYA1$ gene is the human homolog of the Drosophila $EYA1$ gene, which is essential for eye development in this species. In humans, it consists of 16 coding exons and is localized on chromosome 8q13.3. The $EYA1$ protein is essential for branchial arch and ear development. Studies have shown that $EYA1$ homozygous-deficient mice lack ears and kidneys, and $EYA1$ heterozygous-deficient mice present phenotypes resembling BORS. $EYA1$ acts as a protein phosphatase and transcriptional coactivator. The known pathogenic genes of BORS include $EYA1$, SIX1, and SIX5. $EYA1$ gene mutations account for approximately 40% of BOS cases, while SIX5 and SIX1 genes account for 5% and less than 1%, respectively. No distinct relationships were observed between the nature of the mutations and clinical features associated with BORSD. To date, more than 240 pathological mutations in $EYA1$ have been reported. However, in China, the mutations (c. 466C > T, p.Q156X, c. 1735delG, and p.D579fs) were first identified in 2012. With the development of next-generation sequencing technology, an increasing number of novel $EYA1$ mutations have been reported in Chinese BOS/BORS patients (Table 2). These results suggest that the BOR criteria are potential indications for molecular studies on diagnosing $EYA1$-associated syndromes. $EYA1$ is also affected by gene dose effects, and clinical phenotypic heterogeneity may be related to insufficient gene doses of $EYA1$. However, gene activity can be recognized only when the number of encoded proteins exceeds a certain threshold. In addition, gene regulation of specific molecules encoding different amounts of proteins determines the wide phenotypic variation in BOS/BORS patients between or within families. Consequently, we identified the mutation, c. 1697_1698delinsT (p. lys566Ilefs *73), located in exon 17 of $EYA1$, implying that two adjacent bases (AG) mutate to one base (T). Therefore, we classified this mutation as a frameshift mutation. Frameshift or nonsense mutations are the most commonly detected mutations, followed by missense and splice-site mutations. In our study, the frameshift mutation at the site caused the coding amino acid substitution from Lys566 to Ile (p. lys566Ilefs *73), and the deletion of the base further resulted in a subsequent amino acid change, which led to BOS occurrence. The electropherograms obtained by Sanger sequencing showed double peaks with mixed bands (Fig. 4D), and that the 73rd position, followed by Lys566, had became a stop codon.
Table 2
Reported EYA1 mutations in Chinese patients with BOS/BORS

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Location</th>
<th>Mutation(c.)</th>
<th>Protein change</th>
<th>Mutation type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BORS</td>
<td>Exon 7</td>
<td>c.466C &gt; T</td>
<td>p. QGln156X</td>
<td>Missense</td>
<td>17</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 11</td>
<td>c.967A &gt; T</td>
<td>p. Arg323X</td>
<td>Nonsense</td>
<td>4</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 17</td>
<td>c.1735delG</td>
<td>p.D579fs</td>
<td>Frameshift</td>
<td>17</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 15</td>
<td>c.1381delA</td>
<td>p.R461fs467X</td>
<td>Frameshift</td>
<td>2</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 1-18</td>
<td>Entire deletion</td>
<td>Loss protein</td>
<td>CNVs</td>
<td>1</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 1-18</td>
<td>Entire deletion</td>
<td>Loss protein</td>
<td>CNVs</td>
<td>19</td>
</tr>
<tr>
<td>BOS</td>
<td>Exon 16</td>
<td>c.1493_1494insAT</td>
<td>p.Ile498PhefsTer*3</td>
<td>Frameshift</td>
<td>7</td>
</tr>
<tr>
<td>BOS</td>
<td>Intron 10</td>
<td>c.967-2A &gt; G</td>
<td>---</td>
<td>Splicing</td>
<td>7</td>
</tr>
<tr>
<td>BOS</td>
<td>Exon 17</td>
<td>c.1627C &gt; T</td>
<td>p.Gln543Ter</td>
<td>Missing</td>
<td>20</td>
</tr>
<tr>
<td>BOS</td>
<td>Exon 12</td>
<td>c.1075_1077delinsAT</td>
<td>p.Gly359Ilefs*7</td>
<td>Frameshift</td>
<td>21</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 15</td>
<td>c.1425delC</td>
<td>p.As579fs*60</td>
<td>Frameshift</td>
<td>22</td>
</tr>
<tr>
<td>BORS</td>
<td>Exon 10</td>
<td>c.889C &gt; T</td>
<td>p.Arg297*</td>
<td>Nonsense</td>
<td>22</td>
</tr>
<tr>
<td>BORS</td>
<td>Intron 12</td>
<td>c.1140 + 1G &gt; A</td>
<td>---</td>
<td>Splicing</td>
<td>22</td>
</tr>
<tr>
<td>BORS</td>
<td>Intron 11</td>
<td>c.1050 + 1G &gt; T</td>
<td>---</td>
<td>Splicing</td>
<td>22</td>
</tr>
</tbody>
</table>

EYA1, eye absent homolog 1; BOS, branchio-otic syndrome; BORS, branchio-oto-renal syndrome; CNV, copy number variation

Based on the American College of Medical Genetics and Genomics (ACMG) guidelines, the variation observed in our study is a) a frameshift mutation in the EYA1 gene, and loss-of-function is a known disease mechanism (ACMG pathogenicity criteria: PVS1); b) strongly conserved in the position and variant not found in gnomAD (PM2); and c) co-segregated with the disease in multiple affected family members (PP1). Based on this evidence, we assessed this mutation as “pathogenic,” with an important role in the genetic etiology of the EYA1 family.

According to the law of autosomal recessive inheritance, the offspring of the proband are at a 50% increased risk of inheriting biallelic pathogenic variants of EYA1. Therefore, identifying the genetic pathogenic factors for the disease is of great significance. Patients suspected of having BORDS should be screened with the diagnostic criteria, and genetic analysis of peripheral blood DNA should be
conducted with the patient's informed consent. With the emergence of new technologies in recent years, many novel pathogenic genes have been reported, but many unknown pathogenic genes still need to be discovered. The BORDS gene test can be used to assist the diagnosis of diseases and guide prenatal screening and diagnosis. At present, symptomatic treatment is mainly adopted for patients which resolve the problems.

5. Conclusion

We identified a novel heterozygous de novo pathogenic frameshift mutation in the EAY1 gene in a family with BOS and other phenotypes, including BVW and unilateral SOM, which have not been reported previously. Our results expand both the mutational and phenotypic spectra of BOS, suggesting that the mutation of a key amino acid residue is an etiologic factor. Considering the high phenotypic heterogeneity of BORS in patients who are difficult to diagnose based on clinical manifestations alone, genetic testing for candidate pathogenic gene mutations can successfully supplement clinical diagnosis.

Declarations

- Ethical Approval and Consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Second Xiangya Hospital (certificate number: k074).

- Consent for publication

All authors approve the manuscript and give their consent for submission and publication.

- Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- Competing interests

The authors declare no competing interest

- Authors' contributions

Jun He wrote the main manuscript text. Yu Gu and Li jin collect the data. Jihao Ren and Tuanfang Yin prepared figure. Jinfeng Fu prepered the Table. Wei liu design the experiments,provide funding and revise the manuscript. All authors read and approved the final manuscript.

References


**Figures**

![Pedigree of the family](image)

**Figure 1**

Pedigree of the family
Figure 2

Clinical manifestations and audiological examination

A. The proband had bilateral preauricular pits (white arrow [A1–A2]), bilateral branchial fistula (white arrow [A3–A4]), type III cup-shaped ear in the right ear (red arrow [A1]), and external auditory canal stenosis. B. Pure-tone thresholds revealed severe-to-profound sensorineural hearing loss in the left ear
and mixed hearing loss in the right ear (average air conduction threshold, 59 dB; bone conduction threshold, 26 dB). **C.** Acoustic immittance tests showed type “A” tympanogram in the left ear and type “B” tympanogram in the right ear that indicated SOM.

**Figure 3**

**HRCT of proband**

**A.** Cochlea hypoplasia in the right ear (arrow). **B.** Lateral semicircular canal and vestibular fused into a single cavity in the left ear (arrow). **C.** Cochlea hypoplasia with less than two turns in the left ear. **D.** Soft tissue shadow in the right middle ear (red arrow). Cochlea hypoplasia in both side (white arrow). **E.** Lateral semicircular canal malformation together with the vestibular fused into a single cavity (arrow). A, B, and C: coronal view; C and D: axial view. HRCT, high-resolution computed tomography.
Figure 4

WES results of family with BOS

A. Distribution ratio of SNV/InDel sites in different regions and B. of different functional types. C. Bar chart of top 10 gene scores (EYA1 had the highest score). D. Sequencing diagram of the frameshift
variants in the proband. Red arrow indicates change in base position. BOS, branchio-otic syndrome; SNV/InDel, Single Nucleotide Variant (insertion/deletion); WES, whole-exome sequencing