Novel mutations in the *CYP11B2* gene causing aldosterone synthase deficiency

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Abstract. Aldosterone synthase deficiency (ASD) is a rare, autosomal recessive inherited disease. Mutations in the *CYP11B2* gene are responsible for the occurrence of ASD, and the clinical manifestations of ASD vary with age. Affected infants may develop symptoms of mineralocorticoid deficiency, including clinical presentation with frequent vomiting, a variable degree of hyponatremia, hyperkalemia, and metabolic acidosis combined with poor growth, which are easily confused with several other endocrine genopathies, including pseudohypoaldosteronism type 1 and congenital adrenal hyperplasia. In the present study, whole exome sequencing (WES) was used to screen for causal variants in the genome of a Chinese pediatric patient with confusing endocrine disorder symptoms. Clinical symptoms of frequent vomiting, hyponatremia and hyperkalemia were selected as the filtering indices to analyze the WES data. Clinically relevant variants were subsequently verified using Sanger sequencing. Minigene construct analysis was used to assess the consequence of a splicing variant in the *CYP11B2* gene. The compound heterozygous mutations, c.1009C>T and c.240-1G>A, in the *CYP11B2* gene were identified and confirmed, and represented novel variants. Sequence analysis results revealed that the c.1009C>T mutation at codon 337 of exon 6 was a nonsense mutation, which led to early termination of the protein translation process. In addition, further investigation of the splicing pattern in a minigene construct showed that the c.240-1G>A mutation led to the preservation of intron 1, with the 3'-splice site disappearing during transcriptional processing of the mRNA. Using molecular genetic assessments, the patient was finally diagnosed with ASD. Therefore, the present study identified two novel *CYP11B2* gene mutations in a Chinese patient with ASD, indicating exome sequencing as an effective diagnostic tool for rare endocrine-metabolic diseases.

Introduction

Aldosterone is the principal mineralocorticoid hormone in humans, which is synthesized predominantly in the adrenal zona glomerulosa. It regulates sodium excretion and intravascular volume via distal convoluted tubules and renal cortex collecting ducts (1). Aldosterone synthase (CYP11B2), a cytochrome P450 enzyme encoded by the *CYP11B2* gene, is a key enzyme that controls the final three steps of aldosterone biosynthesis (2). The first step is the 11-β-hydroxylation of 11-deoxycorticosterone to corticosterone; the second step is the 18-hydroxylation of corticosterone to 18-hydroxycorticosterone (18OHB), and the third step is the 18-oxidation of 18-hydroxycorticosterone to aldosterone (2). *CYP11B2* (OMIM124080) is located at 8q24.3 and contains nine exons encoding steroid 11-β-hydroxylase, 18-hydroxylase and 18-oxidase. *CYP11B2* exhibits a high level of homology to the *CYP11B1* gene, which encodes the steroid, 11-β-hydroxylase (2-4).

Mutations in the *CYP11B2* gene cause aldosterone synthase deficiency (ASD); the clinical manifestations of which vary with age (2). Affected infants may develop symptoms of mineralocorticoid deficiency, and present clinically with frequent vomiting, a variable degree of hyponatremia, hyperkalemia, and metabolic acidosis combined with poor growth. Older children and adults usually have normal serum electrolytes, even if untreated (1-3). The clinical features of ASD are easily confused with several other endocrine disorders, particularly prior to obtaining definitive biochemical results. These endocrine disorders include pseudohypoaldosteronism type 1 (PHA1), which is characterized by resistance to the actions

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of aldosterone, and congenital adrenal hyperplasia (CAH), associated with 21-hydroxylase deficiency, 11-β-hydroxylase deficiency, or 3-β-hydroxysteroid dehydrogenase deficiency. For the clinical diagnosis of ASD, it may difficult to determine the differential diagnosis from the above-mentioned confusion. In addition, patients may succumb to mortality as a result of salt wasting and high blood potassium (5-8). Therefore, the establishment of a prompt and accurate diagnostic method is required.

The present study describes the clinical features of a 4 month-old boy with the clinical symptoms described above. Using whole exome sequencing (WES) technology, two novel variants of the *CYP11B2* gene were identified, and the functional effects of the mutation were assessed. The pathogenicity of the c.240-1G>A mutation was further investigated by analyzing its effect on splicing in a minigene construct.

Subjects and methods

Clinical description of the patient. A 4-month-old male infant was recruited to the Gastroenterology department of the Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine (Shanghai, China) due to frequent vomiting (6-8 times/day) without inducements, shortly following birth, and had not improved with age. The patient was a full-term baby and had an uncomplicated delivery with a birth weight of 2,960 g. His parents were physical healthy, with non-consanguineous marriage. Physical examination showed his weight was 3,300 g, height was 54 cm, heart rate was 168 bpm, respiratory rate was 42 bpm, blood pressure was 82/55 mmHg, and body temperature was 37.5°C. He had severe malnutrition and moderate dehydration. The patient had male-appearing genitalia, with marginally darkened color scrotal skin, and ~1 ml bilateral testicles. Neither heart murmur, nor hepatosplenomegaly were found. Congenital anomaly of the digestive tract and inborn metabolic diseases were excluded. The patient had initially been treated for gastroesophageal reflux and allergy to formula in another hospital, without a satisfactory improvement. The present study was approved by the ethics committee of the Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine.

The results of the laboratory examinations performed on the first visit to hospital are shown in Table I. Based on the examination results, the baby was treated intravenously for rehydration, supplementation with sodium salt, correction of acidosis and nutritional support. However, it was difficult to correct hyponatremia and hyperkalemia following shifting to oral therapy, even with sodium supplementation to 18 mEq/kg/day. Problems in adrenal hormone production were considered, although the laboratory data did not provide evidence to confirm a diagnosis, and hormone detection can be affected by several factors (9). In order to obtain a clinical diagnosis, mutant genes were screened using WES, and *CYP11B2* gene mutations were identified, however, due to the high levels of aldosterone at the first visit, aldosterone levels were evaluated again. Unlike the first visit, a low aldosterone level (38.12 pg/ml) was found. Subsequently, the patient was simultaneously treated with 9α-fluorohydrocortisone, and electrolyte levels rapidly reached

equilibrium (data not shown). The effectiveness of the treatment supported the genetic diagnosis and the use of molecular genetic assessments, the patient was finally diagnosed with ASD.

WES and data analysis. The steps of the WES experiment were based on the report by Wang J *et al* (10). The genomic DNA of the patient and parents were isolated from 2 ml peripheral blood samples collected from the cubital veins using a QIAamp Blood DNA Mini kit® (Qiagen GmbH, Hilden, Germany). A total of 3 µg DNA from the patient was processed through shearing using a Covarias® M220 Ultrasonicator system (Covaris, Inc. Woburn, MA, USA) to result in sizes of 150-200 bp. An adapter-ligated library was prepared, and enrichment of the coding exons and flanking intronic regions was performed. Clusters were then generated by isothermal bridge amplification using an Illumina cBot station, and sequencing was performed on an Illumina HiSeq 2000 system (Illumina, Inc., San Diego, CA, USA).

Base calling and sequence read quality assessment were performed using Illumina HCS 2.2.58 software (Illumina, Inc.) for the Illumina HiSeq 2000 system which included new versions of HiSeq control software and Real Time Analysis. Alignment of the sequence reads to a reference human genome (Human 37.3; SNP135) was performed using NextGENe® (SoftGenetics LLC, State College, PA, USA). All single nucleotide variants (SNVs) and indels were saved in a VCF format file, and uploaded for Ingenuity® Variant Analysis™ (Ingenuity Systems, Mountain View, CA, USA) for biological analysis and interpretation.

Sanger sequencing verification of the CYP11B2 gene. The primers for amplification of the *CYP11B2* gene (GenBank accession no. NM_000498.3) were designed using UCSC ExonPrimer online software (http://genome.ucsc.edu/index.html) and synthesized by Map Biotechnology, Co., Ltd., Shanghai, China. The primers designed for exon 2 were as follows: Forward 5'-CAGAGAAAACCCCAGCTCAC-3' and reverse 5'-CAACCCACAGTGCAGACG-3'; and the primers designed to amplify exon 6 were as follows: Forward 5'-CCCAAGTGGTCATCAAGGTT-3' and reverse 5'-GGTGTTGAAGAGGGATTCCA-3. The exons and the exon-intron boundaries were amplified using polymerase chain reaction (PCR; Takara Biotechnology, Co., Ltd., Dalian, China). The reaction mixture for each amplification contained 1X Premix Taq (Ex Taq Version 2.0; cat. no. RR003; Takara Biotechnology, Co., Ltd.), 100 ng genomic DNA, and 1 pmol forward and reverse primer in a final volume of 25 µl. The reaction was carried out with the following PCR conditions: Initial denaturation at 95°C for 5 min, then 19 cycles of 95°C for 30 sec, 65°C for 30 sec, 72°C for 45 sec, 14 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and a final elongation step at 72°C for 5 min using a C1000™ Thermal Cycler PCR instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR products (5 µl) were examined on a 1% agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen GmbH). The resulting DNA was sequenced using the ABI3730XL sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the forward and reverse primers. The sequence data were analyzed using Mutation Surveyor® software version 4.0.4 (SoftGenetics, LLC).

Minigene construction. The splice region (c.240-1G>A; intron 1) of the *CYP11B2* gene was amplified from the patient's genomic DNA using the following specific primer pair: Forward 5'-GCgaattcAGAGTCTCAGGCAGGTCCA-3' and reverse 5'-GCggatccGATGTGCTTTTGGGTCCTAC-3'. This was performed using PrimeSTAR HS DNA Polymerase (Takara Biotechnology, Co., Ltd.). The forward and reverse primer sequences were located in the upstream of the promoter region and in intron 2, respectively. The length of the PCR product was 986 bp. The restriction enzyme sites *Eco*RI and *Bam*HI were inserted into the primer sequences to enable directional cloning. DNA from the patient's father who did not carry the genetic variant was used as a control. Target fragments were ligated into the pcDNA3.1/Myc-His B vector (Invitrogen; Thermo Fisher Scientific, Inc.) using solution I ligase (Takara Biotechnology, Co., Ltd.), according to the manufacturer's protocol.

Cell culture and cell transfection. HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO_2 incubator at 37°C. Cell transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Total RNA isolation, cDNA synthesis and splice site analysis. At 48 h post- plasmid transfection, the total RNA from the HEK 293 cells was isolated using an RNeasy Mini kit (Qiagen GmbH), and the RNA samples were treated with 5 U/μl DNase I (Takara Biotechnology, Co., Ltd.). cDNA chains were obtained from 1 μg RNA using C1000™ Thermal Cycler PCR instrument (Bio-Rad Laboratories, Inc.) with a Primer Script™ RT Reagent kit (cat. no. RR037A; Takara Biotechnology, Co., Ltd.). The following primers were used to amplify a 330 bp PCR product for the target cDNA fragment of the *CYP11B2* gene: Forward 5'-GCAGAGGTGTGCGTGGCAG-3' and reverse 5'-CCAGGGCTCCAGGATCATC-3'. The forward and reverse primer sequences were located in exon 1 and exon 2, respectively. Subsequently, the PCR products were visualized by electrophoresis on a 2% agarose gel (Sangon Biotech, Co., Ltd., Shanghai, China) following staining with 0.5 μg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). In addition, the PCR products were sequenced for further identification. *GAPDH* was used as a control for RNA quality, and the following primers were used to amplify a 245 bp PCR product for *GAPDH*: Forward 5'-GTCAGTGGTGGACCTGACCT-3' and reverse 5'-TGCTGTAGCCAAATTCGTTG-3'.

Results

Identification and confirmation of the mutated gene. To obtain a rapid and accurate clinical diagnosis, the patient was screened for causal variants using WES. Exome sequencing yielded a total of 101,306,626 reads, and the mean target coverage was 136 reads with 95.48% having 20x coverage, and 99.71% having 1x coverage. The candidate variants were first screened with criterion of a minor allele frequency under 3% in the 1000 Genomes Project, the NHLBI exome variant server, or in

Table I. Laboratory results during initial hospital visit.

Factor	Value	Normal range
Na (mmol/l)	113.00	137.00-145.00
K (mmol/l)	6.30	3.50-5.10
CL (mmol/l)	77.00	98.00-107.00
Lactic acid (mmol/l)	2.70	0.70-2.10
Aldosterone (pg/ml)	361.00	50.00-313.00
PRA (ng/ml/h)	0.03	0.13-1.94
Cortisol (μg/dl)	26.90	5.70-16.60
ACTH (pg/ml)	33.00	8.00-80.00
17-hydroxyprogesterone (nmol/l)	1.10	<30.00

PRA, plasma renin activity; ACTH, adrenocorticotropic hormone.

Table II. Results of the second set of laboratory examinations, obtained in the outpatient department.

Factor	Value	Normal range
Aldosterone (pg/ml)	29.00	50.00-313.00
Cortisol (μg/dl)	6.60	5.70-16.60
ACTH (pg/ml)	10.00	8.00-80.00
Na (mmol/l)	137.40	137.00-145.00
K (mmol/l)	4.70	3.50-5.10
CL (mmol/l)	105.00	98.00-107.00

ACTH, adrenocorticotropic hormone.

50 HapMap control exomes, the area of analysis area included each exon and ~20 bp of exon-intron boundaries. Subsequently, clinical symptoms of frequent vomiting, hyponatremia, and hyperkalemia were chosen as the filtering indexes to analyze the candidate variants. Ultimately, the results demonstrated that the patient had compound heterozygous mutations in the *CYP11B2* gene, which were considered to contribute to the patient's condition. Of the two mutations, one was a heterozygous point transition (c.1009C>T) in exon 6. Sequence analysis results showed that the c.1009C>T mutation at codon 337 of exon 6 was a nonsense mutation, which led to early termination of the protein translation process (p.Q337X). The other was a transversion of guanine to adenine (c.240-1G>A) in the first base of the 3'-splice site of intron 1 (Fig. 1). Further analysis revealed that c.240-1G>A was an intronic variation, which was involved in the agGT-aaGT splicing site, which occurs at a highly conserved 'ag-GT' site of the intron-exon junction region. The two mutations were identified as novel mutations, according to the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk). In addition, several genes associated with the clinical symptoms mentioned above are wild-type, including *CYP11B1*, *CYP17A1*, *CYP21A2*, *3βHSD*, *SCNN1A*, *SCNN1B*, *SCNN1 G*, *NR3C2*, *DAX1*.

To assess the identified variations in the patient's parents, and further confirm the results of the WES, the proband and parents were examined using PCR Sanger sequencing.
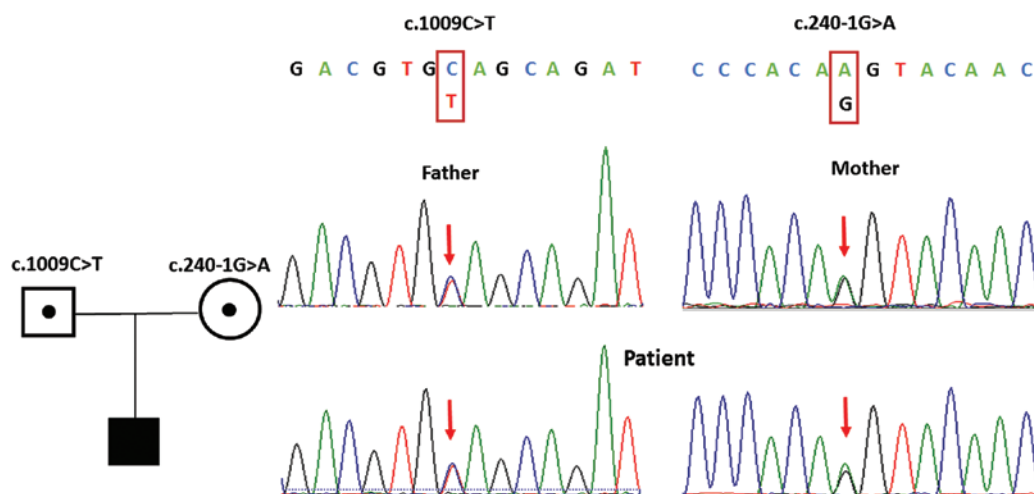
Figure 1. Identification of two novel mutations in the *CYP11B2* gene. Sequences showed compound heterozygous mutations (c.1009C>T, p.Q337X in exon 6 and c.240-1G>A in intron 1) in the patient. Red arrows, mutant bases.
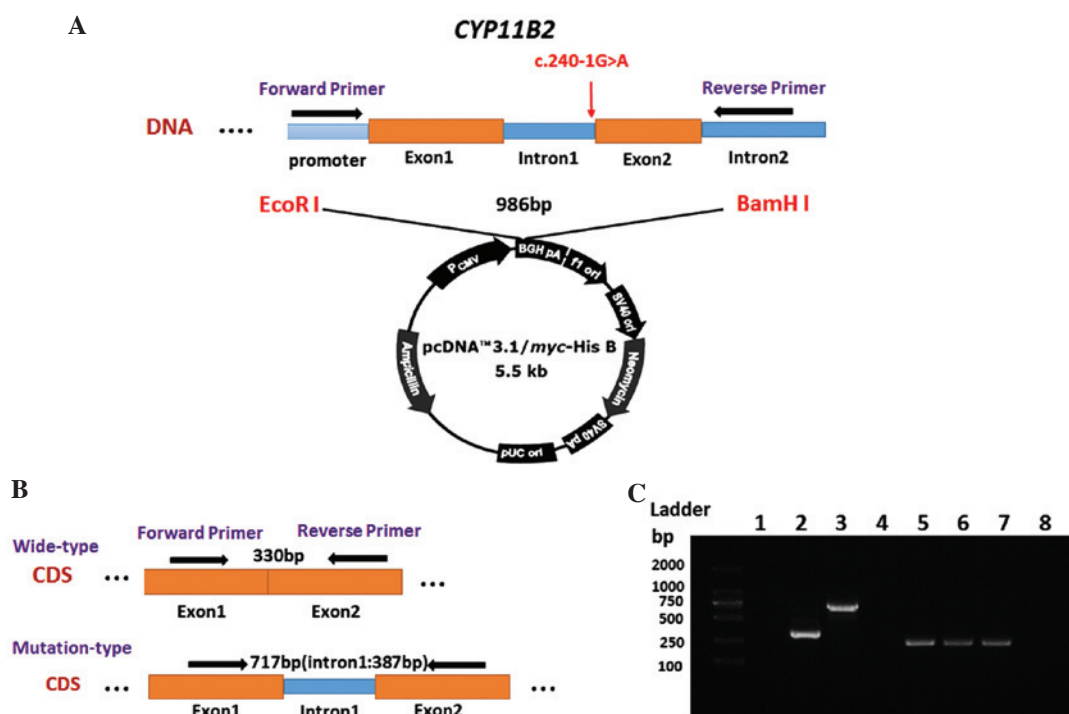

Figure 2. Splicing assays of the c.240-1G>A mutation. (A) Schematic representation of the mutant-type plasmid construct. The splice region (c.240-1G>A, intron1) of the *CYP11B2* gene was amplified, and the products were ligated into the pcDNA3.1/Myc-His B vector. (B) Schematic representation of expected RT-PCR results in HEK 293 cells transfected with different plasmids. The c.240-1G>A mutant type produced a longer band. (C) Electrophoresis was used to analyze the RT-PCR results. Lane 1, empty pcDNA3.1 vector; lane 2, wild-type *CYP11B2* (330 bp); lane 3, c.240-1G>A mutant (717 bp); lanes 4 and 8, ddH$_2$O (negative control, lanes 5, 6, and 7, GAPDH (control; 245 bp). RT-PCR, reverse transcription polymerase chain reaction.

Direct sequencing results revealed that the patient's father was heterozygous for the c.1009C>T and p.Q337X mutation, and his mother was heterozygous for the c.240-1G>A mutation, which indicated that the gene mutations in the pediatric patient were inherited from her parents. These results led to the conclusion that these two novel compound heterozygous mutations in the *CYP11B2* gene were the genetic cause for the patient's condition.

Splicing assays of the c.240-1G>A mutation. To evaluate the effect of the c.240-1G>A mutation on splicing, a minigene construct was generated, which was transiently transfected into cultured HEK 293 cells (Fig. 2A and B). The HEK 293 cells were transfected with either a mutant-type *CYP11B2*, wild-type *CYP11B2* or empty pcDNA3.1 vector (negative control). As shown in Fig. 2C, a difference in the size of the cDNA fragment was observed between the wild-type and mutant-type *CYP11B2,* as expected. The wild-type *CYP11B2* produced a PCR product of ~330 bp, whereas the mutant-type construct produced a longer band of 717 bp. *GAPDH* was used as a control for RNA quality and quantity. Clone sequencing verified that the mutant *CYP11B2* construct contained the

387 nucleotides, which constituted intron 1. Therefore, it was concluded that the c.240-1G>A mutation of the 3'-splice site at the intron-exon boundary resulted in the retention of intron 1 in the RNA.

Discussion

ASD has been classified into two phenotypes. Type 1 ASD is defined as a total loss of enzyme activity caused by a *CYP11B2* mutation. Patients with Type 1 ASD have low to normal levels of 18OHB, undetectable to low levels of plasma aldosterone and an increased B/18OHB ratio, whereas type 2 ASD is reported to involve only defects in 18-hydroxylase and 18-oxidase activities, but not in 11-β-hydroxylase activity, and serum levels of aldosterone are usually within normal limits, particularly in older children and adults. Affected individuals are often characterized by an increased 18OHB/aldosterone ratio (2,11-14). The predominant method of treatment for patients with ASD is mineralocorticoid and sodium supplementation (15). During the course of treatment, clinicians gradually adjust the dose according to levels of serum electrolytes and hormones, particularly potassium (15). When treatment is effective, patients with ASD are able to maintain a satisfactory clinical and metabolic state, without salt-wasting crisis, and exhibit normal growth and development (16).

To date, ~49 mutations have been identified in the *CYP11B2* gene, including missense/nonsense mutations, splicing mutations, regulatory mutations, small insertions/deletions, gross deletions and complex rearrangements (data from HGMD). The proportion of missense/nonsense mutations accounts for ~70% (33/49). ASD is a rare, autosomal recessive disease, and cases have been identified in the Iranian Jewish, European and North American populations. It has also been reported in Asian populations, including Thai, Japanese, and Indian individuals (8,14,15). However, to the best of our knowledge, only one Chinese patient with ASD has been reported with a heterozygous missense mutation (c.977C>A) and a heterozygous small deletion mutation (c.523_525delAAG) of the *CYP11B2* gene (17). In the present study, two mutations (c.1009C>T and c.240-1G>A) in the *CYP11B2* gene were successfully identified and, using familial analysis, it was confirmed that the patient was a compound heterozygote, with the two mutations confirmed as novel mutations. Sequence analysis indicated that the c.1009C>T (p.Q337X) mutation on exon 6 resulted in a truncate protein, which may be due to premature stop codon formation or nonsense-mediated RNA decay. The c.240-1G>A mutation occurred at the conserved ag-GT site of the intron-exon boundary region, and minigene construction analysis revealed that the mutation resulted in aberrant splicing, in which intron 1 was preserved and the 3'-splice site of intron 1 disappeared.

Following a review of the patient's hormone levels a second time, the first set of results may have been a result of the stress response resulting from the child's poor health condition, which introduced complexity in clinical judgment. The second set of examination results in the outpatient department showed that the electrolyte balance was well-controlled (Table II). The identified nonsense mutation and intron retention mutation were expected to result in two nonfunctional alleles, however,

the serum levels of aldosterone indicated that aldosterone synthase remained, indicating that the mutations may have affected only the somatic cells of the adrenal gland, and the aldosterone synthesis catalyzed by the *CYP11B2* gene in other systems, including the subcapsular aldosterone-producing cell clusters are involved in the compensatory effect (18,19).

It is known that the precondition of effective treatment is a definitive clinical diagnosis (20). The high efficiency of conventional Sanger sequencing is based on defined clinical manifestations in line with expectations, and it may not apply to the diagnosis of polygenic diseases or complicated rare diseases (21). It is difficult to distinguish ASD from several other endocrine genopathies, including PHA1 and CAH, particularly following hormone therapy in the affected individuals. In addition, it is difficult to survey the actual hormone levels in a 4-month-old infant, which is critical for clinical diagnosis and treatment. The present study demonstrated that WES, which is a type of next generation sequencing, may be an effective and rapid detection technology for certain complicated rare diseases.

In the present study, two novel mutations were identified in the *CYP11B2* gene in a Chinese pediatric patient with ASD. The c.1009C>T mutation was a nonsense mutation, which led to a premature stop codon (p.Q337X). The pathogenicity of the c.240-1G>A mutation was further demonstrated by analyzing its effect on splicing in a minigene construct, which resulted in the preservation of intron 1, and the disappearance of the 3'-splice site during post-transcriptional processing of the mRNA. The present study not only enhances our understanding of the *CYP11B2* mutation, but also demonstrated that WES may serve as a powerful tool for the clinical diagnosis of complex diseases.

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