Clinical and genetic analysis of cytochrome P450 oxidoreductase (POR) deficiency in a female and the analysis of a novel POR intron mutation causing alternative mRNA splicing

Overall analysis of a female with POR deficiency

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Abstract

Objective To characterize the clinical features of a female with P450 oxidoreductase (POR) deficiency and to investigate the underlying mechanisms of POR inactivation.

Methods The proband was a 35-year-old woman with primary infertility and menstrual irregularity. The reproductive endocrine profile was evaluated. DNA sequencing was conducted for the identification of *POR* gene mutation. RT-PCR was performed to confirm the impact of the mutation on *POR* mRNA. A molecular model was built for the structural analysis of mutant POR protein.

Results The evaluation of reproductive endocrine profile revealed elevation of serum follicle-stimulating hormone (11.48 mIU/ml), progesterone (11.00 ng/ml), 17 α -hydroxyprogesterone (24.24 nmol/l), dehydroepiandrosterone (6300 nmol/l), and androstenedione (3.89 nmol/l) and decreased estradiol (36.02 pg/ml). Sequencing of the *POR* gene showed the female was a compound heterozygote of the paternal P399_E401 deletion and a novel maternal IVS14-1G>C mutation. Functional analysis revealed IVS14-1G>C mutation caused alternative splicing of *POR* mRNA, with the loss of 12 nucleotides in exon 15 (c.1898_1909delGTCTACGTCCAG). Also, the resulting mutant POR protein had a V603_Q606 deletion, which inactivated the nucleotide-binding domain of NADPH in POR protein (K602_Q606).

Conclusion The mutation IVS14-1G>C of the *POR* gene could cause alternative splicing of *POR* mRNA and dysfunction of the resulting POR protein. Under proper IVF strategy with glucocorticoid therapy and endometrial preparation, females with mild POR deficiency still have the opportunity to have a live birth.

Keywords Cytochrome P450 oxidoreductase deficiency · Live birth · In vitro fertilization · Alternative splicing · Compound heterozygote

Introduction

Cytochrome P450 oxidoreductase (POR) is a flavoprotein that transfers electrons from reduced nicotinamide adenine

dinucleotide phosphate (NADPH) to all microsomal cytochrome P450 enzymes including steroidogenic enzymes 17 α -hydroxylase/17,20 lyase (CYP17A1), aromatase, 21-hydroxylase (CYP21A2), and the principal hepatic drug-metabolizing cytochrome P450 enzymes [1] (Fig. 1a). POR also plays the role of electron donor for some non-P450 enzymes, with squalene monooxygenases [2], fatty acid elongase [3], heme oxygenase [4], and cytochrome b5 (CYB5A) [5] included.

The X-ray crystal structure of rat POR shows that POR protein has two lobes (Fig. 1b): one binds flavin adenine dinucleotide (FAD) domain containing the NADPH-binding site, while the other binds flavin mononucleotide (FMN) domain, from which electrons flow to the heme iron of the P450 enzymes for catalysis reaction [6]. Dramatic conformational

Précis

Clinical and genetic analysis of POR deficiency in a female.

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Fig. 1 Schematic of the role of POR in adrenal steroidogenesis biosynthesis pathway and the interaction between reduced nicotinamide adenine dinucleotide phosphate (NADPH), POR, and microsomal cytochrome P450 enzymes. **a** POR plays a key role in steroidogenesis biosynthesis through the transfer of electrons from NADPH to all cytochrome P450 enzymes including CYP17A1, aromatase, and CYP21A2. **b** POR has two lobes: one binds flavin adenine dinucleotide (FAD) domain containing the NADPH-binding site, while the other binds flavin mononucleotide (FMN) domain, from which electrons flow to the heme iron of the P450 enzymes. The interaction between NADPH and POR leads to electron transfer from NADPH to FAD moiety, triggering a

conformational change in POR to allow FAD and FMN coming into close apposition, so that the electrons pass from the FAD domain to the FMN domain. Then, POR protein conformation returns to its original state to allow FMN domain of POR interacting with the redox partner binding site of the P450 enzyme. Besides, the heme-containing protein cytochrome b5 (CYB5A) facilitates allosteric interaction of the POR and the P450 enzyme, leading to the enhancement of electron flux. POR, cytochrome P450 oxidoreductase; CYP17A1, 17 α -hydroxylase/17,20 lyase; CYP21A2, 21-hydroxylase; P450aro, aromatase; DHEA, dehydroepiandrosterone

changes would take place during the process of electron transfer. In this process, the electron transfer from NADPH to FAD moiety will trigger a conformational change in POR, allowing FAD and FMN coming into close apposition, so that electrons can transfer from the FAD to the FMN. After that, POR conformation restores to the initial state and allows the FMN domain to interact with P450 enzymes [7]. The human *POR* gene (NCBI accession: NG_008930; MIM: 124015) located on chromosome 7q11.2 consists of 16 exons including 15 protein-coding exons and a first untranslated exon that lies 38.8 kb upstream and initiates transcription [8].

The significant role of POR in multiple biochemical functions suggested that the dysfunction of POR could cause severe clinical manifestations. Moreover, embryonic lethality was observed in *POR*-knockout mice [9, 10]. However, *POR* deficiency (OMIM ID: 201750) was compatible with human life. Patients with *POR* deficiency showed multiple clinical manifestations including a

severe skeletal malformation syndrome termed Antley-Bixler syndrome (ABS), adrenal insufficiency, disordered steroidogenesis, and disordered sex development [11–15]. Most of these clinical features have been well documented, but to the best of our knowledge, investigations regarding infertility associated with *POR* mutations are still rare [16, 17].

We report the case of a Chinese female who manifested primary infertility and menstrual irregularity due to *POR* deficiency caused by a compound heterozygous mutation in the *POR* gene (p.399_401delPSE on the paternal allele and IVS14-1G>G/C on the maternal allele). A successful live birth of a normal male/female infant was achieved through in vitro fertilization frozen-thawed embryo transfer (IVF-FET) using hormone replacement therapy (HRT) for endometrial preparation and glucocorticoid therapy. A novel mutation IVS14-1G>G/C of the *POR* gene was identified, and functional analysis revealed this mutation caused alternative splicing of *POR* mRNA.

Materials and methods

Case report

A 35-year-old Chinese female visited the reproduction medicine center of a large tertiary center with primary infertility and menstrual irregularity. This female had a height of 161 cm, a weight of 60 kg, and a BMI of 23.1. The blood pressure was normal. Physical examination showed Tanner stage IV breast development with no galactorrhea, sparse axillary, and pubic hair. Examination of her genitalia indicated hypoplastic labia majora. Her baseline serum reproductive endocrine profile (Table 1) showed elevation of serum follicle-stimulating hormone (FSH) (11.48 mIU/ml), progesterone (11.00 ng/ml), 17 α -hydroxyprogesterone (24.24 nmol/l), dehydroepiandrosterone (DHEA) (6300 nmol/l), and androstenedione (3.89 nmol/l) and low estradiol (36.02 pg/ml). The serum levels of luteinizing hormone (LH) (7.53 mIU/ml), testosterone (1.39 nmol/l), prolactin (2.50 ng/ml), cortisone (419 nmol/l), and adrenocorticotrophic hormone (ACTH) (30 pg/ml) were normal. No skeletal malformation, dysmorphism, or limited limb movements were observed, and X-rays showed no radioulnar or radiohumeral synostosis. Follicular monitoring by a series of transvaginal ultrasounds revealed multiple follicular developments and ovulatory dysfunction. The serum levels of estradiol remained low (29–87.3 pg/ml) during follicular monitoring. The ovulation could be induced by the administration of human chorionic gonadotrophin (HCG). The patient had a laparoscopic right ovarian cystectomy and genital anaplasty because of aberrant genitalia, stenosis of the vagina, and multiple ovarian cysts 6 years ago.

During the IVF treatment process, this female received glucocorticoid therapy (prednisone 5 mg t.i.d.) to suppress serum progesterone. The patient underwent controlled ovarian stimulation according to the routine long GnRH agonist protocol described elsewhere [18]. Briefly, pituitary suppression was achieved by daily subcutaneous injection of triptorelin acetate (Decapeptyl; Ferring) starting at the mid-luteal phase of the preceding cycle until the HCG day. After that, recombinant follicle-stimulating hormone (rFSH) (Gonal-f, Merck Serono, Switzerland) and highly purified urinary gonadotropin (hMG, Lizhu, China) were administered for ovarian stimulation. The peak estrogen value was 190 pg/ml. HCG (250 mcg; Ovidrel; Serono) was given to trigger ovulation. Thirty-six hours later, 8 mature oocytes were retrieved through transvaginal follicular aspiration guided by ultrasound under anesthesia. The methods for sperm preparation, IVF, and embryo culture have been reported previously [19]. Four embryos were obtained and two reached the blastocyst stage, all of which were cryopreserved for subsequent FET cycles due to serum progesterone elevation. The FET cycles were managed as hormone replacement treatment cycles described previously [18]. Two blastocysts were transferred in the first FET cycle, which ended with biochemical pregnancy. Clinical pregnancy was achieved in the second FET cycle with the remaining two embryos transferred. In the 12th week of gestation, the female's blood pressure increased, with a peak of 150/100 mmHg. Diagnosis of chronic hypertension in pregnancy was made. Blood pressure was controlled with the administration of calcium channel antagonist (diltiazem; Sanofi) 60 mg bid. No other complication was found during the pregnancy. By 34 weeks and 3 days of gestation, a cesarean section was performed, and the live normal male and

Table 1 Biochemical characteristics of the proband

Plasma steroid/pituitary hormone	Results	Reference
FSH (mIU/ml)	11.48	Follicular, 3.85–8.78; ovulatory, 4.54–22.51; luteal, 1.79–5.12
LH (mIU/ml)	7.53	Follicular, 2.12–10.89; ovulatory, 19.18–103.03; luteal, 1.20–12.86
Estradiol (pg/ml)	36.02	Follicular, 24–114; ovulatory, 62–534; luteal, 80–273
Progesterone (ng/ml)	11	Follicular, 0.31–1.52; luteal, 5.16–18.56
Testosterone (nmol/l)	1.39	< 2.6
PRL (ng/ml)	2.5	3.34–26.72
ACTH (pg/ml)	30	25–100
COR (nmol/l)	419	135–650
DHEA-S (nmol/l)	6300	3540 $\pm$ 1310
AN (nmol/l)	3.89	Follicular, 2.7 $\pm$ 1; luteal, 5.2 $\pm$ 1.5
17OH-Pro (nmol/l)	24.24	Follicular, 1.3 $\pm$ 0.25; luteal, 7.4 $\pm$ 2.0

FSH, follicle-stimulating hormone; *LH*, luteinizing hormone; *PRL*, prolactin; *ACTH*, adrenocorticotrophic hormone; *COR*, cortisone; *DHEA-S*, dehydroepiandrosterone sulfate; *AN*, androstenedione; *17OH-Pro*, 17 α -hydroxyprogesterone

female newborns were delivered with a weight of 2.3 kg and 2.2 kg, respectively.

DNA isolation and sequence analysis

Peripheral blood samples were collected in EDTA-K2 tubes from the patient and her family members. The study protocol was approved by the Medical Ethics Committee of Tongji Hospital. Written informed consent was obtained from all participants. Research was conducted according to the Declaration of Helsinki for medical research.

Genomic DNA was extracted from peripheral blood using the QG-Mini80 workflow with the DB-S kit (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions. *POR* consists of 16 exons spanning 71.7-Kb region on chromosome 7q11.23. We designed PCR primers to amplify and sequence all coding exons and adjacent noncoding regions in the *POR* gene using the Primer Premier 6.0 software. Specific primers in the intronic regions flanking the 15 exons of *POR* were designed on the basis of the National Center for Biotechnology Information (NCBI) sequences GI 4508114 and GI 11181841 (Table 2). The PCR products were directly sequenced with BigDye Terminator v3.1 on the 3130 × 1 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Finally, Chromas and DNAMAN programs were used to identify mutations by two independent investigators.

Splicing assay

Lymphocytes were extracted from peripheral blood, and RNA was isolated from lymphocytes using the RNeasy Mini Kit of QIAGEN (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To prevent contamination of genomic DNA, each sample was treated with RNase-free DNase I (Fermentas). Reverse transcription was performed following a standard protocol using PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China) and random Oligo dT Primer (TaKaRa, Dalian, China). Primers spanning exons 13, 14, 15, and 16 (RT-PCR) were:

sense : 5'-CATAGGCTTCATCCAGGAG-3';
anti-sense : 5'-CGATGTCGTAGAAGGTGTT-3'.

PCR amplification was performed for the exon 13 to exon 16 cDNA fragments according to a standard protocol using SYBR Premix Ex Taq (TaKaRa, Dalian, China). The PCR products were subjected to TA subcloning for sequencing following a standard protocol of pEASY-T1 Simple Cloning Kit (Transgen, Beijing, China). Briefly, the resulting DNA fragments were cloned into pEASY™-T1 Simple Cloning Vector and then the plasmids were transfected into Trans1-T1 Phage Resistant Chemically Competent Cell (Transgen,

Table 2 Primers for PCR and sequencing

Exon	Sequence (5' → 3')
POR-E1F	ACCACGCACTTTCATTCTCTG
POR-E1R	ATCAGCTCTAGGGGAAGGGC
POR-E2F	CTCCTACCCCGTGCACTGAC
POR-E2R	TCACCCCAAAATGCTACAAGG
POR-E3F	GGAGCCCTGGTGTGGATTA
POR-E3R	GCTTTGGTTAGGCAAGAATGACT
POR-E4F	GACTCAAAGCCAGGAAGGAAAG
POR-E4R	TGGGTTTGGTTTGGGAGATG
POR-E5F	CCACGACACTCAGACATCCCT
POR-E5R	CCTTTAGTCTCCAGCACCCAA
POR-E6F	TTCAGTGGCCAGTGTTCT
POR-E6R	CATAAACCCAGAGCATCAGGAAG
POR-E7F	GGTGACAGTCCTGAGCTTTG
POR-E7R	GACGGAGTGTCTTCTAACCTTGC
POR-E8F	TGCTTCTTGTCGTATGTACCTGG
POR-E8R	TGCAGAGTAAGGTGGCTAAGTGA
POR-E9-10F	CTCTGAGATTCCCTGTGCTTTGT
POR-E9-10R	GCCTAAGCAGAAGCTCAACCC
POR-E11-12F	AGCTGGCCCAAGGTGTACAC
POR-E11-12R	ATCTCGGAGCTCCTGTGCC
POR-E13F	AAGAGGCCCTGGGTGAGTG
POR-E13R	CCGTAGTACAGCAGCGTCTCC
POR-E14-15F	ACCCCTTCATAGGCTTCATCCAG
POR-E14-15R	CTCAGCCACGATGTCGTAGAAG
POR-E16F	CATCTACGTCTGTGGGTGAGTGA
POR-E16R	GTTAAGTTGATGCAGGTGGAGGT

E, exon; F, forward; R, reverse

Beijing, China). Positive clones were screened out by the culture in liquid medium containing ampicillin and kanamycin and subjected to direct sequencing.

Molecular modeling

The X-ray crystal structure of human *POR* protein (PDB: 3QE2) was used as the template for three-dimensional modeling of the location and structural impact of the studied *POR* mutations. ΔVYVQ mutant structure was constructed by using SWISS-MODEL [20].

Results

DNA sequence analysis

Genetic analysis of the *POR* gene in this proposita and her family revealed two kinds of mutations: (1) p.399_401delPSE in exon 11, c. 1195_1203delCCTCGGAGC from paternal

line; (2) IVS14-1G>G/C in intron 14 on the maternal allele, which is located at the wildtype splice acceptor site (AG) of exon 15 (Fig. 2a). Her father and elder sister carried a heterozygous mutation of p.399_401delPSE in exon 11. Her mother and younger sister had a heterozygous mutation of IVS14-1G>G/C in intron 14. All other family members showed no clinical manifestation (Fig. 2b).

Analysis of alternative splicing

Figure 3 shows the effects of IVS14-1G>C on *POR* mRNA splicing. TA subcloning and sequencing of the RT-PCR products revealed that IVS14-1G>C caused the inactivation of the original splice acceptor site in intron 14 and the utilization of cryptic splice acceptor site in exon 15, which led to the loss of 12 nucleotides of exon 15 (c.1898_1909delGTCTACGTCCAG) and a V603_Q606 deletion in the resulting mutant *POR* protein. Also, the sequence of control was identical to the reference sequence, with no alternative splicing

Comparison of ΔVYVQ mutant modeling structure and wildtype *POR*

ΔVYVQ mutant structure was constructed by using SWISS-MODEL [20], highly similar to wildtype *POR*, which possessed FAD and FMN domains. However, as shown in Fig. 4 (Fig. 4a, b), it did not have the NADP domain. Compared with the *POR* structure, the conformation was more flexible within the missing VYVQ portion, and it expanded up to almost 9 Å from b to c, indicating that VYVQ was vital to maintain the NADP domain. Within the NADP domain, Y604 and Q606 were found to bind to NADP via

hydrogen bond (Fig. 4c, d). Furthermore, aromatic ring of Y604 bound strongly to adenine moiety of NADP via π - π conjugation. π - π conjugation was believed to be important in stabilizing the protein structure [6, 21, 22]. Lack of VYVQ, especially Y604 and Q606, contributed to more disordered conformation and NADP missing in the mutant structure.

Discussion

Cytochrome P450 oxidoreductase acts as a key regulator in steroidogenesis as well as the hydroxylation of fatty acids, prostaglandins, exogenous toxins, and drug metabolism. Therefore, patients with *POR* deficiency had multiple clinical manifestations [23]. Also, there have been reports of patients with *POR* deficiency who were phenotypically normal adults with infertility, similar to our case [11–13, 15]. However, the studies on fertility treatments and successful pregnancy of these patients are rare [16, 17].

This female's basal state of reproductive endocrine profile (Table 1) showed an elevation levels of serum progesterone (11.00 ng/ml), 17 α -hydroxyprogesterone (24.24 nmol/l), and DHEA (6300 nmol/l). The possible causes (Fig. 1) included CYP17A1 deficiency, aromatase deficiency, *POR* deficiency, or the heme-containing protein CYB5A deficiency. DNA sequence analysis revealed that there were mutations in the *POR* gene, while no mutations were found in other genes. Then, we performed a genetic analysis of the *POR* gene in her family to confirm this conclusion (Fig. 2).

The impact of serum estradiol level near the HCG day on IVF outcomes has been studied for over 25 years. Many

Fig. 2 Genetic analysis of *POR* gene and pedigree of this family.

a The patient carried two kinds of mutations including p.399_401delPSE in exon 11 which came from paternal line and IVS14-1G>G/C in intron 14 which was inherited from maternal line. **b** Her father and elder sister carried a heterozygous mutation of p.399_401delPSE in exon 11. Her mother and younger sister had a heterozygous mutation of IVS14-1G>G/C in intron 14. All other family members showed no clinical manifestation

Fig. 3 Effects of the POR IVS14-1G>C mutation on splicing of POR mRNA and protein structure. Novel splice acceptor site due to IVS14-1G>C results in the mutant transcript which loses 12 nucleotides of exon 15 (c.1898_1909delGTCTACGTCCAG), compared with the correctly spliced normal mRNA

studies revealed that increasing serum estradiol during ovulation induction was associated with favorable pregnancy outcomes [24, 25]. Also, increasing oocyte retrieval and improved embryo quality were reported in IVF cycles with high serum estradiol levels [26, 27]. Additionally, low serum estradiol levels during ovulation induction were regarded as the indications of compromised IVF outcomes by some studies [28, 29]. Severe estradiol deficiency might be related to the arrest of follicular growth [30]. However, many retrospective studies failed to show this correlation [24–27, 31–33]. They suggested the serum estradiol concentration alone near the HCG day being a poor predictor of IVF outcomes. In our

report, this female exhibited low estradiol levels during the therapy process. But mature oocytes and high-quality embryos could still be obtained using assisted reproductive technology. This fact suggested that high levels of serum estradiol might not be necessary for oocyte development and IVF success.

The inverse relationship between serum progesterone elevation near HCG day and clinical pregnancy rates has been reported [34–37]. High serum progesterone levels could impair endometrial receptivity [34, 35, 38]. The excessive non-cyclic progesterone level might be the main cause of infertility in females with POR deficiency [39]. Therefore, glucocorticoid

Fig. 4 NADP-binding sites in POR and its comparison with Δ VYVQ mutant modeling structure. **a** Δ VYVQ mutant modeling structure. It is generated by using SWISS-MODEL possessing FAD domain as well as FMN domain but lacks NADP domain. **b** Structural comparison of POR and Δ VYVQ mutant. Compared with wildtype, the mutant has minor structure change colored in red rectangle but has a distensible space which

is enlarged up to almost 9 Å from b to c. The mutant is colored in green, wildtype colored in cyan. **c** NADP-binding sites in POR. Aromatic ring of Y604 has strong aromatic stacking interaction with adenine moiety of NADP. Y604 and Q606 both bind to NADP via hydrogen bond. **d** POR structure in surface (colored in light-blue). Y604 is colored in green, Q606 colored in red

therapy was used to suppress serum progesterone. Also, the “freeze-all” strategy in which embryos were frozen and transferred in subsequent frozen-thawed cycles was developed to bypass impaired endometrial receptivity due to progesterone elevation [40]. In our report, IVF followed by frozen embryo transfer under glucocorticoid suppression therapy was conducted for this female.

Since the first description of POR deficiency in 2004 [41], more than 50 *POR* mutations have been reported. However, no patients with two apparently null mutations have been reported so far, indicating a complete loss of *POR* function might be incompatible with life [12, 13]. In order to understand the molecular basis of *POR* mutations, we conducted a genetic analysis of the female and her family members. DNA sequencing revealed this proband was compound heterozygosity of a paternal P399_E401 deletion in exon 11 and a maternal IVS14-1G>C in intron 14. *POR* mutation P399_E401del was first reported in two unrelated Turkish families in 2011 [42], both of which were homozygotes with manifestations of Antley-Bixler syndrome and disorder of sexual development but subclinical cortisol deficiency. In vitro functional studies of P399_E401del *POR* protein showed that catalytic efficiency of 21-hydroxylation of progesterone was reduced by 68%, 17 α -hydroxylation of progesterone by 76%, 17,20-lyase action on 17OH-pregnenolone by 69%, aromatization of androstenedione by 85%, and cytochrome c reduction activity by 80%. Besides, protein structure analysis suggested P399_E401del would disturb *POR* protein structure stability and the electron transfer from NADPH to FAD.

Our analysis of the *POR* mRNA extracted from the proband's peripheral blood leukocytes revealed that IVS14-1G>C mutation caused the abandon of this splice site and the utilization of cryptic splice acceptor site in exon 15, which led to the loss of 12 nucleotides of exon 15 (c.1898_1909delGTCTACGTCCAG) and a V603_Q606 deletion in the resulting mutant *POR* protein. Furthermore, the mutant amino acid sequence analysis demonstrated that lack of VYVQ, especially Y604 and Q606, contributed to more disordered conformation and NADP missing in the mutant structure (Fig. 4), which would damage the interaction between *POR* and NADPH. Thus, there would be a decline of *POR* enzyme activity as the interaction between *POR* and NADPH was the basis of the *POR* protein function. However, more studies are needed to test our hypothesis even further.

In conclusion, *POR* deficiency is a unique congenital adrenal hyperplasia caused by mutations in the *POR* gene, which leads to impaired steroidogenesis and infertility. However, females with mild *POR* deficiency still have the opportunity to have live normal birth under proper IVF strategy of glucocorticoid therapy and endometrial preparation. Our research suggested that the compound heterozygote in the current case could not encode fully functional *POR* protein, which might disturb the interaction between *POR* and cytochrome P450

enzymes. Also, a novel mutation IVS14-1G>G/C of the *POR* gene was identified. Functional analysis revealed that IVS14-1G>C mutation could cause alternative splicing of *POR* mRNA and the dysfunction of the resulting *POR* protein.

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Conflict of interest The authors declare that they have no competing interests.

Authors' contributions Tao Zhang carried out the analysis and interpretation of data and writing of the manuscript. Zhou Li, Bo Huang, and Xinling Ren have been involved in the ultrasound examination and critical manuscript revisions. Lei Jin and Guijin Zhu participated in its design and coordination and helped to draft the manuscript. Wei Yang participated in the conception and design of the study. All authors have read and approved the final version of the manuscript.

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