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Novel NR5A1 Missense Mutation in Premature Ovarian Failure: Detection in Han Chinese Indicates Causation in Different Ethnic Groups

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Abstract

Background: The etiology of most premature ovarian failure (POF) cases is usually elusive. Although genetic causes clearly exist and a likely susceptible region of 8q22.3 has been discovered, no predominant explanation exists for POF. More recently, evidences have indicated that mutations in NR5A1 gene could be causative for POF. We therefore screened for mutations in the NR5A1 gene in a large cohort of Chinese women with non-syndromic POF.

Methods: Mutation screening of NR5A1 gene was performed in 400 Han Chinese women with well-defined 46,XX idiopathic non-syndromic POF and 400 controls. Subsequently, functional characterization of the novel mutation identified was evaluated in vitro.

Results: A novel heterozygous missense mutation [c.13T>G (p.Tyr5Asp)] in NR5A1 was identified in 1 of 384 patients (0.26%). This mutation impaired transcriptional activation on Amh, Inhibin-a, Cyp11a1 and Cyp19a1 gene, as shown by transactivation assays. However, no dominant negative effect was observed, nor was there impact on protein expression and nuclear localization.

Conclusions: This novel mutation p.Tyr5Asp, in a novel non-domain region, is presumed to result in haploinsufficiency. Irrespective, perturbation in NR5A1 is not a common explanation for POF in Chinese.

Introduction

Premature ovarian failure (POF), also termed primary ovarian insufficiency (POI), refers to cessation of normal ovarian function before the age of 40 years. Approximately 1% of the population has POF prior to age 40 and only 0.1% or less before age 30 [1,2]. The etiology of POF is highly heterogeneous. Chromosomal abnormalities account for 12% of cases [3], and the familial aggregation often associated with POF indicates a genetic contribution. Causative mutations in several genes (e.g., NOBOX, FIGLA, BMP15 and GDP5) have been identified in non-syndromic POF [4–8], but none of these genes are perturbed in more than a small minority of POF cases, in a given ethnic group. More recently, our genome-wide association study (GWAS) of POF discovered a significantly susceptible region of 8q22.3; however, it was concluded as an important yet undefined long distance regulatory region affecting oogenesis [9]. Therefore, the underlying explanation for POF remains largely unknown (idiopathic).

Nuclear receptor subfamily 5, group A, member 1 (NR5A1, MIM#184757), at human chromosome 9q33, encodes steroidogenic factor 1 (SF1), a nuclear receptor involved in adrenal and gonadal development, steroidogenesis, and reproduction [10]. SF1 serves as a master transcriptional regulator of multiple genes, including STAR, CYP11A1, CYP19A1, AMH, INHA, and LHB [11–15]. Human SF1 protein is mainly expressed in ovarian somatic cells of growing follicles and corpus lutea [16]. Granulosa cell-specific NR5A1 knockout mice exhibit infertility, hypoplastic ovaries with decreased follicles and absence of corpora lutea [17]. These findings indicate a critical role of NR5A1 in ovarian development and function.

In humans, NR5A1 mutations are associated with a wide range of phenotypes. Heterozygous, or rarely homozygous, variants were
described in 46,XY disorders of sex development (DSD), with (rare) or without adrenal insufficiency, and milder phenotypes–hypospadias, cryptorchidism, anorchidia, and male infertility [18]. A pathogenic role of NR5A1 in 46,XX POF has also been observed. Lourenco et al. [19] reported 19 different mutations in 4 families in which affected members had 46,XY DSD or 46,XX POF, and 2 out of 25 (8.0%) sporadic POF cases. In contrast, subsequent cohort studies demonstrated only a minor contribution of NR5A1 to POF in several different populations [20–23]. Janse et al. found 1.4% in New Zealander (mutation carriers from Asian, Caucasian and Mediterranean); Lakhal et al. found no mutations in Tunisian but Philibert et al. found 3.8% in Tunisian. And more recently Voican et al. reported the mutation rate to be 1.6% in French.

Given these discrepant results, we screened the NR5A1 gene in a large cohort of Chinese women with POF. Our cohort is the largest collected in any ethnic group and distinct in being exclusively Han Chinese. We found one novel heterozygous missense mutation, which was shown to impact functional significance with impaired transactivation activity.

Methods

Patients

A total of 400 unrelated Han Chinese women with POF were recruited from Center for Reproductive Medicine, Shandong Provincial Hospital Affiliated to Shandong University between April 2003 and June 2012. Inclusion criteria included primary amenorrhea (PA) or secondary amenorrhea (SA) for at least 6 months prior to the age of 40 years and two measures of serum FSH >40 IU/L obtained at least 1 month apart. In this cohort, 35 presented with PA and 365 with SA. A positive family history was considered if another first- or second-degree female family member had POF or early menopause (menopause before 45 years old). Patients with karyotypic abnormalities, previous chemo/radio-therapy, ovarian surgery or autoimmune diseases known to induce POF were excluded. Women with accompanying somatic anomalies were sought and excluded, particularly any reported as associated with syndromic POF (e.g., blepharophimosis-ptosis-epicanthus inversus syndrome, neurosensory deafness and cerebellar ataxia). Four hundred Han Chinese females with regular menses and normal hormone level were enrolled as controls.

Ethics Statement

The study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University. Written informed consent was obtained from each subject.

Mutation Screening of NR5A1 Gene

Genomic DNA was extracted from peripheral blood according to standard protocols. The entire coding sequence (exon 2–7) and intron-exon boundaries of NR5A1 gene (NM_004959.4) were PCR amplified and directly sequenced (ABI 3730xl DNA Analyzer, Applied Biosystems, Foster City, CA). Novel variants identified were confirmed by bidirectional sequencing from another two independent PCR products. Nomenclature of variants identified was established according to Human Genome Variation Society (HGVS, www.hgvs.org/mutnomen). Primers and PCR conditions are available upon request.

Plasmids Construction

SF1 expression vector containing p.Y5D mutation was generated by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit; Stratagene, LaJolla, CA) with wild-type (WT) mouse SF1 expression vector as a template (a generous gift from Prof. Jameson). The luciferase reporter vectors containing SF1-responsive minimal promoters of murine Amh, murine Inhibin-a, murine Cyp1a1, and rat Cyp19a1 in pGL3-basic (Promega, Madison, WI, USA) were constructed. For expression and cellular localization, mouse Nr5a1 cDNA was amplified and cloned in-frame into pEGFP-C3 expression vector (Clontech, Mountain View, CA), resulting in a fusion protein with a GFP tag at its N-terminus (pEGFP-C3-Sf1). The p.Y5D mutation was introduced as described above. All constructs were validated by sequencing. Primers used are detailed in Table S1.

Transactivation Activity Assays

Transactivation activity assays were performed in 48-well plates using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) and a Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA). Human embryonic kidney HEK293T cells were transiently co-transfected with empty (Mock), wild-type (WT), or mutant (MT) SF1 expression vectors with reporter constructs described above. To further assess the possible dominant negative effect, the WT vector with either empty vector or the mutant (1:1) was transfected with each reporter. In addition, increasing mutant vectors (0, 10, 20, 30, 40 ng) in the absence or presence of constant WT (10 ng) and Amh-pGL3 reporter (540 ng) were co-transfected. Total transfected plasmids were adjusted with empty vectors. Renilla reporter pRL-TK (Promega) was used as an internal control. Cells were lysed 24 hours later, and luciferase activity was measured with a luminometer reader (Enspire, PerkinElmer). Results were normalized against Renilla luciferase activity.

Expression and Nuclear Localization

HEK293T cells were transfected either with empty vector (Mock), WT or with mutant pEGFP-C3-Sf1 expression vector. Twenty-four hours after transfection, nuclear counterstaining was performed with Hoechst 33342 (Beyotime Institute of Biotechnology, Jiangsu, China), and then visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistics

Results in transactivation activity assays were expressed as percentage of WT activity (100%) and represent the Mean±S.D. of 3 independent experiments, each performed in triplicate. Statistical significance was examined using Student’s t-test. Chi-square test was used for comparison of allele frequencies. A P value <0.05 was considered statistically significant.

Results

A Novel Missense Mutation Identified within Gene NR5A1

The clinical characteristics of the 400 patients studied are summarized in Table 1. Direct sequencing of NR5A1 gene was successful in 384 POF patients. A novel heterozygous missense mutation c.13T>G (p.Tyr5Asp, p.Y5D) in exon 2 was identified in one individual. This variant was absent in dbSNP database or 400 Chinese control women. The mutated tyrosine, located seven residues upstream of the first zinc finger DNA-binding domain (DBD), was highly conserved among SF1 orthologs (Fig. 1).

The single p.Y5D carrier developed spontaneous menarche at the age of 19, but experienced irregular menses and subsequent secondary amenorrhea 1 year later. Transvaginal ultrasonography showed hypoplastic uterus and non-detectable ovaries. FSH was significantly elevated (78.3 IU/L), while estradiol was undetectable. No other family member had POF or a form of 46,XY DSD.
Two known single nucleotide polymorphisms (SNPs), rs1110061 and rs2297605, were also identified. However, the allele frequencies of these SNPs in our POF cohort were not statistically different from those in the Asian population (P > 0.05) (Table S2).

Impaired Transactivation Activity of the p.Y5D Mutant

Transactivation activity assay showed that the p.Y5D mutation impaired the transactivation activity on different SF1 responsive promoter reporters, including Amh, Inhibin-a, Cyp11a1 and Cyp19a1 (all P < 0.01) (Fig. 2A, B, C, D). Because of the heterozygous status of the patient, the dominant negative effect of the mutant over WT protein was assessed. Co-transfection of mutant with WT did not interfere with the transactivation of WT protein either when transfected in a 1:1 ratio (Fig. 2A, B, C, D), or when increasing mutants transfected with a fixed WT even in ratio 5:1 (Amh only) (Fig. 2E).

No Impact of the p.Y5D Mutation on Protein Expression and Nuclear Localization

To assess the impact of the p.Y5D mutation on protein expression and nuclear localization, we generated GFP-tagged Sf1 WT and mutant expression vectors and transfected them into HEK293T cells. The WT showed exclusively strong nuclear localization with relative nucleolar exclusion. A similar pattern was seen for the mutant (Fig. 3).

Discussion

In our large cohort of Chinese patients known to have well-defined 46,XX non-syndromic POF, a novel heterozygous missense mutation was identified (1/384, 0.26%). The c.13T>G transition located in exon 2, outside any classic domains of SF1, resulted in a p.Tyr5Asp mutation. The highly conserved tyrosine residue was located adjacent to the first zinc finger of DBD, a region that contributed to specific recognition and interaction with promoter responsive elements in target genes. In vitro transactivation assays using Amh, Inhibin-a, Cyp11a1 and Cyp19a1 promoters showed impaired transactivation activity of the p.Y5D mutant, and thus confirmed its deleterious effect. However, a dominant negative effect was not observed (Fig. 2), consistent with previous reports [22,24,25].

The two SNP, rs1110061 and rs2297605, detected in our study had drawn attention of others. Rs1110061 (Gly146Ala) was reported to be associated with POF in recent cohorts of Tunisian women [21,22]. Nevertheless, previous functional assays showed inconsistent results, that is, Gly146Ala resulted in either a decrease of 20–35% or no impact on the transactivation activity on target gene promoters [22,23,26]. Still, given that no significant difference existed in allele frequency between our POF cohort

Table 1. Clinical characteristics of 400 Chinese women with POF.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean±S.D./N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (yr)*</td>
<td>29.82±4.95</td>
</tr>
<tr>
<td>Age at menarche (yr)*</td>
<td>14.85±2.20</td>
</tr>
<tr>
<td>Age of amenorrhea (yr)*</td>
<td>24.39±5.86</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>76.09±29.82</td>
</tr>
<tr>
<td>E2 (pmol/L)</td>
<td>25.38±42.42</td>
</tr>
<tr>
<td>Family history</td>
<td>41(10.3%)</td>
</tr>
<tr>
<td>Parental consanguinity</td>
<td>4(1.0%)</td>
</tr>
</tbody>
</table>

*Refer to patients with secondary amenorrhea.

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and the general Asian population, we considered rs1110061 to be only a polymorphism not conferring susceptibility to POF.

POF is genetically heterogeneous probably attributed to the complex genetic networks modulating human folliculogenesis and oogenesis. Ovarian phenotypes of Nr5a1 null mice made it an excellent candidate gene for POF in human. NR5A1/SF1 regulates the transcription of CYP11A1, CYP19A1, AMH and INH-A genes involved in steroidogenesis, ovarian development and function. Dysregulation of any protein encoded by these genes could lead to ovarian dysfunction. Disruption of CYP11A1 (P450 Side Chain Cleavage, P450sc) or CYP19A1 (Aromatase) could contribute to steroid hormones deficiency, estrogen in particular, and subsequent abnormal folliculogenesis. Firm evidence indicated an inhibitory role of AMH in initiation of primordial follicle growth and FSH responsiveness of small growing follicles [27,28]. As an important negative regulator of pituitary FSH and paracrine factor in folliculogenesis, INHIBIN-A is also a downstream target of NR5A1 [29]. It is plausible that a decline in AMH and inhibin levels would result in increased recruitment of primordial follicles, raised FSH or FSH responsiveness of small growing follicles, increased FSH-dependent cyclic recruitment, and hence, premature exhaustion of ovarian reserve occurred ultimately [27,29]. However, we cannot exclude the possibility that other regulatory pathways, even other genes, and/or environmental factors might participate in ovarian pathogenesis.

Figure 2. Transactivation activity assay. Co-transfection of empty (Mock), wild-type (WT), or mutant (MT) SF1 expression vectors and a Amh (A), Inhibin-a (B), Cyp11a1 (C) or Cyp19a1 (D) promoter reporter were performed in HEK293T cells. Results are expressed as a percentage of WT activity (RLU WT%). The potential dominant negative effect of the p.Y5D mutant was assessed by co-transfecting WT expression vector with empty or MT vector (1:1) (A, B, C, D) and increasing MT (0, 10, 20, 30, 40 ng) with 10 ng empty (−) or 10 ng WT (+) vector (Amh only) (E) in HEK293T cells. ** P<0.01, *** P<0.001. RLU, relative light units. doi:10.1371/journal.pone.0074759.g002

Figure 3. Expression and nuclear localization of p.Y5D mutant. HEK293T cells were transiently transfected with empty vector (Mock), wild-type (WT) or mutant (MT) pEGFP-C3-SF1 expression vector. The nuclei were counterstained with Hoechst33342 (blue). Scale bar = 10 um. doi:10.1371/journal.pone.0074759.g003
Table 2. Molecular and phenotypic features of 46,XX sporadic premature ovarian failure (POF) cases with NR5A1 mutations.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Mutation rate (%)</th>
<th>Sequence variation*</th>
<th>Amino acid variation*</th>
<th>Location</th>
<th>Functional effect</th>
<th>Dominant negative</th>
<th>Age at diagnosis (yrs)</th>
<th>Ethnicity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA(N=35) SA(N=365)</td>
<td>1 (0.3)</td>
<td>c.13T&gt;G</td>
<td>p.Y5D</td>
<td>Impaired transactivation on Amh, Inhibin-a, Cyp1la1 and Cyp19a1 promoters</td>
<td>No</td>
<td>20</td>
<td>Chinese</td>
<td>Current</td>
<td></td>
</tr>
<tr>
<td>PA(N=37) SA(N=143)</td>
<td>3 (1.6)</td>
<td>c.162C&gt;A</td>
<td>p.S54R</td>
<td>DBD</td>
<td>No effect on DNA-binding capacity and transcriptional activity</td>
<td>No</td>
<td>20</td>
<td>Portugal</td>
<td>[23]</td>
</tr>
<tr>
<td>c.593C&gt;T</td>
<td>p.P198L</td>
<td>Hinge</td>
<td>No effect on DNA-binding capacity and transcriptional activity</td>
<td>No</td>
<td>33</td>
<td>North African</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.368G&gt;C; 386C&gt;T</td>
<td>p.G123A; P129L</td>
<td>Hinge</td>
<td>No effect on DNA-binding capacity and transcriptional activity</td>
<td>No</td>
<td>25</td>
<td>North African</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA(N=26)</td>
<td>1 (3.8)</td>
<td>c.763C&gt;T</td>
<td>p.R255C</td>
<td>LBD</td>
<td>Sharp decrease in transactivation on Cyp1la1 and Amh promoters</td>
<td>No</td>
<td>25</td>
<td>Tunisian</td>
<td>[22]</td>
</tr>
<tr>
<td>PA(N=25)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tunisian</td>
<td>[21]</td>
</tr>
<tr>
<td>N=2</td>
<td>1</td>
<td>c.704C&gt;T</td>
<td>p.P235L</td>
<td>LBD</td>
<td>80% of WT transcriptional activity on CYP17A1 and CYP11A1 promoters</td>
<td>No</td>
<td>15 (increased LH&amp;FSH, undetectable E2&amp;AMH, prepubertal uterus, nondetectable ovaries)</td>
<td>Swiss</td>
<td>[25]</td>
</tr>
<tr>
<td>SA(N=356)</td>
<td>5 (1.4)</td>
<td>c.407C&gt;T</td>
<td>p.P136L</td>
<td>Hinge</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Asian, Caucasian</td>
<td>[20]</td>
<td></td>
</tr>
<tr>
<td>c.574G&gt;T</td>
<td>p.A192F</td>
<td>Hinge</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Mediterranean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.575C&gt;T</td>
<td>p.A192V</td>
<td>Hinge</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Mediterranean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.593C&gt;T</td>
<td>p.P198L</td>
<td>Hinge</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Caucasian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.938G&gt;A</td>
<td>p.R313H</td>
<td>LBD</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Caucasian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N=25</td>
<td>2 (8.0)</td>
<td>c.368G&gt;C; 386C&gt;T</td>
<td>p.G123A; P129L</td>
<td>Hinge</td>
<td>Severe loss of activation on CYP11A1 and CYP19A1 promoters (P129L)</td>
<td>Not tested</td>
<td>12.5 (no development of breasts or pubic hair, fibrous ovary without follicles, high FSH = 76 U/L)</td>
<td>Senegalese</td>
<td>[19]</td>
</tr>
<tr>
<td>c.691_699del</td>
<td>p.L231_L233del</td>
<td>LBD</td>
<td>Severe loss of activation on CYP11A1 and CYP19A1 promoters</td>
<td>Not tested</td>
<td>4 month (hypertrophic clitoris, high FSH = 44 U/L)</td>
<td>Italian</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table only refers to novel non-synonymous mutations and all mutations are heterozygous.
PA: primary amenorrhea; SA: secondary amenorrhea; DBD: DNA binding domain; LBD: ligand binding domain.
doi:10.1371/journal.pone.0074759.t002
Table 3. Molecular and phenotypic features of premature ovarian failure (POF) cases in 46,XY DSD families with NR5A1 mutations.

<table>
<thead>
<tr>
<th>Proband</th>
<th>Other affected family members</th>
<th>Sequence variation*</th>
<th>Amino acid variation*</th>
<th>Location</th>
<th>Functional effect</th>
<th>Dominant negative</th>
<th>Ethnicity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>46,XY complete gonadal dysgenesis</td>
<td>Mother: SA at 35 yrs</td>
<td>c.666delC</td>
<td>p.N222fs</td>
<td>Hinge</td>
<td>Severe loss of activation on CYP11A1 and CYP19A1 promoters</td>
<td>Not tested</td>
<td>European</td>
<td>[19]</td>
</tr>
<tr>
<td>46,XY partial gonadal dysgenesis</td>
<td>Sister: SA at 16 yrs</td>
<td>c.3G&gt;A</td>
<td>p.M1I</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>French</td>
<td>[19]</td>
</tr>
<tr>
<td>46,XY DSD</td>
<td>Mother: SA at 29 yrs</td>
<td>c.390delG</td>
<td>p.P131fs</td>
<td>Hinge</td>
<td>Not tested</td>
<td>Not tested</td>
<td>French</td>
<td>[19]</td>
</tr>
<tr>
<td>46,XY DSD</td>
<td>Mother: SA at 32 yrs</td>
<td>c.938G&gt;A</td>
<td>p.R313H</td>
<td>LBD</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Argentine</td>
<td>[31]</td>
</tr>
<tr>
<td>46,XY DSD</td>
<td>Mother's sister: regular menses, high FSH&gt;40 U/L</td>
<td>c.938G&gt;A</td>
<td>p.R313H</td>
<td>LBD</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Argentine</td>
<td>[32]</td>
</tr>
<tr>
<td>46,XY DSD</td>
<td>Mother's mother: menopause at 38 yrs</td>
<td>c.768delC</td>
<td>p.D257fs</td>
<td>LBD</td>
<td>Decrease in transactivation on CYP11A1 and CYP19A1 promoters</td>
<td>No</td>
<td>Japanese</td>
<td></td>
</tr>
</tbody>
</table>

*All mutations are heterozygous except for c.877G>A (p.D293N).

46, XY DSD: 46, XY disorder of sex development; PA: primary amenorrhea; SA: secondary amenorrhea; LBD: ligand binding domain.

doi:10.1371/journal.pone.0074759.t003
46,XX POF, a heterozygous NR5A1 mutation could be inherited from a 46,XX mother either with POF or apparently normal ovarian function, to the 46,XY DSD offspring in a sex-limited dominant manner, which was kind of similar to X-linked inheritance [18,19,31,32] (Table 3). This information would be valuable in counseling even for sporadic cases with POF, because women with a heterozygous NR5A1 mutation are at potential risk of passing it on to her 46,XY offspring.

In summary, we identified a single novel heterozygous NR5A1 mutation in a cohort of Chinese patients with 46,XX non-syndromic POF. This mutation, located in non-domain region, was transcriptionally impaired without a dominant negative effect. Although NR5A1 perturbations appear to be a less common explanation for POF, our identifying a causative mutation, in an ethnic group (Han Chinese) different from that of other reports of mutations, strengthens the case for NR5A1 playing a crucial role in ovarian development and function in some women. Comprehensive researches on signaling pathways associated to NR5A1 in ovary are warranted to demonstrate its plausible causative roles in human POF.

References

Supporting Information
Table S1 Primers used in plasmids construction. (DOCX)
Table S2 Known single nucleotide polymorphisms (SNPs) identified in our POF cohort. (DOCX)

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Author Contributions
Conceived and designed the experiments: ZJC. Performed the experiments: XJ YYQ GYL SDZ. Analyzed the data: XJ YYQ GYL LY JLM. Wrote the paper: XJ. Clinic diagnosis and blood collection: XJ YYQ GYL SDZ. Contributed reagents/materials/analysis tools: LY JLM ZJC. Performed the experiment: XJ YYQ GYL SDZ. Data interpretation: XJ YYQ GYL LY JLM ZJC. Wrote the first draft of the paper: ZJC.

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Competing Interests
The authors have declared that no competing interests exist.

Supporting Information
Table S1 Primers used in plasmids construction. (DOCX)
Table S2 Known single nucleotide polymorphisms (SNPs) identified in our POF cohort. (DOCX)