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GENETICS

A novel *FOXL2* mutation in two infertile patients with blepharophimosis–ptosis–epicanthus inversus syndrome

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Abstract

Background Blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) is a rare, autosomal dominant disease. There are two clinical types of BPES: type I patients have eyelid abnormalities accompanied by infertility in affected females, while type II patients only display eyelid malformations. Previous studies have reported that the forkhead box L2 (*FOXL2*) gene mutations cause BPES.

Purpose To identify plausible *FOXL2* mutation in a Chinese family with BPES and infertility

Methods Mutational screening of *FOXL2* was performed in the affected members and 223 controls. Functional characterization of the novel mutation identified was carried out in vitro by luciferase reporter assay and subcellular localization experiment.

Results A novel heterozygous mutation c.188 T > A (p.I63N) in *FOXL2* was identified in two BPES patients in this family. The mutation abolished the transcriptional repression of FOXL2 on the promoters of *CYP19A1* and *CCND2* genes, as shown by luciferase reporter assays. However, no dominant-negative effect was observed for the mutation, and it did not impact FOXL2 protein nuclear localization and distribution.

Conclusions The mutation c.188 T > A (p.I63N) in *FOXL2* might be causative for BPES and infertility in this family and further amplified the spectrum of *FOXL2* mutations.

Keywords Blepharophimosis–ptosis–epicanthus inversus syndrome · *FOXL2* · Mutation · Infertility

Introduction

Blepharophimosis–ptosis–epicanthus inversus syndrome (BPES; MIM 110100) is a rare autosomal dominant disease with prevalence < 1/50,000 (1). It has been reported that there were no differences in its prevalence based on sex, race, or ethnicity (2). There are two phenotypes of BPES. While type I BPES patients manifest eyelid abnormalities accompanied by infertility in affected females, type II patients only present eyelid malformations and both male and female patients are fertile (3). Mutations in forkhead transcription factor gene *FOXL2* were firstly identified in both types of BPES (4). Recently, a large cohort study identified pathogenic *FOXL2* mutations in about 67% of the BPES patients (5).

FOXL2 is a single-exon gene on human chromosome 3q22.3 which encodes a protein of 376 amino acids containing a 110-amino acid DNA-binding forkhead domain (FHD) and a polyalanine tract of 14 residues (6). The transcription factor encoded by *FOXL2* is selectively expressed in mesenchyme of developing eyelids as well as fetal ovary (4, 7). FOXL2 is the earliest known marker of ovarian differentiation (7). *Foxl2* knockout mice not only displayed craniofacial malformations but also female infertility with follicles blocked between the primordial and primary stages and subsequently undergoing atresia (8, 9). In adult ovary, FOXL2 is expressed in the undifferentiated granulosa cells in small and medium follicles and affects the granulosa cells proliferation, differentiation, and steroidogenesis by repressing the expression of multiple downstream genes, such as *CYP11A1*, *CYP19A1*, and CCND2 (10, 11).

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More than 200 mutations in the *FOXL2* gene have been reported in individuals with BPES (12). Most of the genetic defects identified in BPES were caused by intragenic mutations, and others were caused by genomic rearrangements involving the *FOXL2* gene (13). It was reported that there was a genotype–phenotype correlation, i.e., type I BPES was associated with mutations resulting in a protein truncated upstream of polyalanine sequence, and mutations leading to an extended protein might cause type II BPES (14). But multiple mutations described later did not conform to this correlation (14). Then, it was more generally proposed that complete loss-of-function mutations led to type I BPES with premature ovarian failure (POF), whereas hypomorphic alleles only affect eyelid development and result in type II BPES without ovarian dysfunction (15, 16). However, the genetic and clinical heterogeneity still exit in BPES patients (17, 18), and it needs more efforts to clearly define the relationship between *FOXL2* mutations and BPES types.In the present study, we reported a novel *FOXL2* heterozygous mutation identified in two infertile sisters from a Chinese BPES family and further demonstrated its relationship with sterility of the BPES patients.

Materials and methods

Patients

A non-consanguineous Chinese family with BPES members (Fig. 1A) was recruited from the Reproductive Hospital Affiliated to Shandong University. The proband (II-1) in this family was an infertile female who presented with the typical features of BPES including blepharophimosis, ptosis, and epicanthus inversus from birth. Her younger sister (II-2) also showed a similar eyelid alteration and suffered infertility, while her mother (I-1) and younger brother (II-3) did not have abnormal phenotypes. Although we could not obtain the blood sample from their father (I-2), it was speculated that this disease in this family was inherited from the father who manifested BPES phenotypes. These two sisters (II-1 and II-2) have nonobstructive bilateral fallopian tubes with no history of pelvic surgery, radiation, or autoimmune diseases, and their husbands' semen were normal. The proband had undergone three IVF-ET cycles, but the most oocytes retrieved are unmatured. At the third cycle, there are no mature oocytes even though cultured at embryo laboratory for 48 h. Embryologists believe that the patient egg maturation is an obstacle.

The clinical characteristics of the two sisters with BPES are shown in Table 1. A total of 223 females with regular menses, three or more antral follicles remaining in bilateral and normal hormone levels, were enrolled as controls. The study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University. Written informed consent was obtained from each subject.

Mutational analysis

Genomic DNA was obtained from the leukocytes of peripheral venous blood using QI Aamp DNA Blood Kits (Qiagen, Hilden Germany). The single exon and exon–intron boundaries of FOXL2 gene (NG-012454) were amplified by

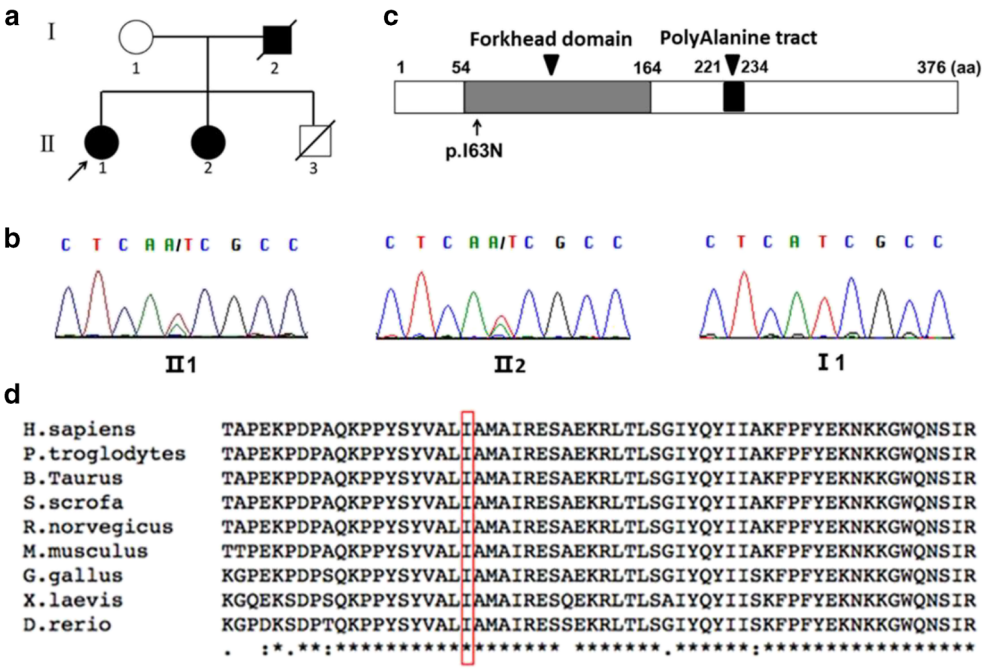

Fig. 1 FOXL2 mutation identified in a BPES family with infertility. (A) Pedigree of the BPES patients, ascertained through II1. (B) FOXL2 gene sequencing was performed in II1, II2, and I1. While the proband (II1) and her sister (II2) carried the mutation c.188 T > A (p.I63N), their mother was normal. (C) Schematic presentation of the location of the *FOXL2* mutation associated with BPES. (D) Sequence alignment of FOXL2 among species with isoleucine residue highlighted and highly conserved

Table 1 Clinical characteristics of the two patients with BPES

Characteristics	II1	II2
Age at diagnosis (yr)	36	33
Age at menarche (yr)	13	15
Age of AUB (yr)	24	–
Basal FSH (IU/l)	5.38	5.51
Basal E_2 (pg/ml)	27.8	22.4
Total AFC	12	8
AMH(ng/ml)	1.472	1.402

AUB abnormal uterine bleeding; *FSH* follicle-stimulating hormone; E_2 estradiol; *AFC* antral follicle count; *AMH*, anti-Müllerian hormone

polymerase chain reaction (PCR) with two overlapping sets of primers: sense primer FOXL2-1F (5'-GCAGTCTGGCTTCC TCAACAA-3′), antisense primer FOXL2-1R (5'-AGGGGACAAAGAGGAGCGAC-3′), sense primer FOXL2-2F (5'-CTGCGAAGACATGTTCGAGAAG-3′), and antisense primer FOXL2-2R (5'-GGACAAAG AGGAGCGACAGG-3′). The PCR products were purified, labeled by BigDye (Terminator v 3.1 Cycle Sequencing Kits, Applied Biosystems, Foster City, CA), and sequenced by ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Novel variants were confirmed by bidirectional sequencing from another two independent PCR runs. Nomenclature of the mutation identified was established according to Human Genome Variation Society (HGVS, http://www.hgvs.org/mutnomen/).

Bioinformatic analysis

The amino acid conservation analysis was performed by using multiple sequence alignment by ClustalW (https://www.genome.jp/tools-bin/clustalw). The possible impact of the amino acid alteration on the function of FOXL2 protein was predicted using the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), the Sorting Tolerant from Intolerant (SIFT) algorithm (http://sift.jcvi.org/www/SIFT_enst_submit.html), and the MutationTaster (http://www.mutationtaster.org/).

Construction of the plasmids

The coding sequence of the *FOXL2* gene was cloned into pcDNA3.1 and pEGFP-N1 vectors. The mutant plasmids carrying p.I63N were generated by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit; Stratagene, LaJolla, CA) with wild-type *FOXL2* expression vectors as templates. The promoters of rat *Cyp19a1*, murine *Cyp11a1*, and human *CCND2* were constructed into pGL3-basic (Promega, Madison, WI, USA) as the luciferase reporter vectors. All constructs were validated by Sanger sequencing.

Luciferase reporter assay

Transcriptional activity assays were performed in 24-well plates. Human embryonic kidney (HEK) 293 cells were transfected by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with empty vector pcDNA3.1, wild-type or mutant *FOXL2* expression vectors with reporter constructs described above. Five groups including (1) 25 ng pcDNA3.1, (2) 25 ng *FOXL2* vector, (3) 25 ng mutant *FOXL2* vector, (4) 12.5 ng pcDNA3.1 plus 12.5 ng wild-type *FOXL2* vector, and (5) 12.5 ng wild-type plus 12.5 ng mutant *FOXL2* vectors, together with 270 ng luciferase reporter plasmids (either pGL3-*CYP19A1*, pGL3-*CYP11A1*, or pGL3-*CCND2*) and10 ng pRL-TK (Promega) plasmid, were transiently co-transfected into HEK293 cells. The total DNA content was maintained at 305 ng/well. Cells were lysed after 36 h post transfection, and the luciferase activities were measured with a luminometer reader (Enspire, PerkinElmer) according to the protocol of Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Results were normalized against Renilla luciferase activity. The experiments were repeated three times.

Subcellular localization experiment

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with the plasmids of empty vector pEGFP-N1, pEGFP-FOXL2, or pEGFP-FOXL2-p.I63N. Twenty-four hours after transfection, the nuclei were counterstained with Hoechst 33258 (Beyotime Institute of Biotechnology, Jiangsu, China), and then the cells were visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Results in transactivation activity assays were expressed as mean ± SD of three independent experiments. One-way analysis of variance (ANOVA) or Student's *t* test were used to compare luciferase activities between different groups. Statistical analyses were performed using the SPSS statistical package version 17.0 (SPSS Inc., USA). P value < 0.05 was considered statistically significant.

Results

A novel missense mutation identified in the *FOXL2* gene

The clinical characteristics of the two females II1 and II2 are summarized in Table 1. A novel heterozygous missense mutation c.188 T > A (p.I63N) was identified in the *FOXL2* gene in II1 and II2 BPES patients, but not in their mother I1 (Fig.

1B). Sanger sequencing confirmed that the variant was absent in 223 control women. The mutated Asparagine was localized in the Forkhead domain, and the position was highly conserved among species (Fig. 1C and D). This amino acid change was probably damaging predicted by PolyPhen-2 (score = 1.000) and SIFT (score = 0.000) programs.

Impaired transcriptional activity of p.I63N mutation

FOXL2 can inhibit the promoter activity of target genes, such as *CYP19A1*, *CYP11A1*, and *CCND2*. To determine whether p.I63N mutation affects the transcriptional repression activity of FOXL2 toward the three genes, mutant *FOXL2* was co-transfected with reporter plasmids carrying *CYP19A1*, *CYP11A1*, or *CCND2* promoters. Luciferase reporter assays showed that mutant FOXL2 significantly impaired the repression activity on the *CYP19A1* and *CCND2* promoters ($P < 0.05$, Fig. 2A and C) but did not significantly change the effect on the *CYP11A1* promoter ($P > 0.05$, Fig. 2B). Furthermore, co-transfection of mutant with wild-type *FOXL2* showed no dominant-negative effect on *CYP11A1*, *CYP19A1*, and *CCND2* promoters (Fig. 2A-C).

No impact of p.I63N mutation on protein expression and nuclear localization

To further investigate the effect of the mutation p.I63N on the protein expression and subcellular location of the FOXL2 protein, pEGFP-N1 plasmids carrying wild-type or mutant FOXL2 were transfected into HEK293 cells. The wild-type FOXL2 protein showed localization in the nucleus in a diffused manner with relative nucleolar exclusion (Fig. 3), consistent with its function as a transcription factor. A similar pattern was seen for the mutant protein p.I63N (Fig. 3).

Discussion

In this study, we reported a novel heterozygous mutation c.188 T > A (p.I63N) within the *FOXL2* gene in a BPES pedigree. The variant was located in the forkhead domain and changed the amino acid which is highly conserved among species. In vitro target gene promoter assay demonstrated that the mutation abolished the inhibitory effects of FOXL2 on *CYP19A1* and *CCND2* promoters. However, no dominant-negative effect was observed, and the subcellular localization of FOXL2 protein was not changed.

As previously reported, genetic defects involving the *FOXL2* gene can be identified in nearly 90% of BPES patients (6). Therefore, we screened the *FOXL2* gene in a BPES pedigree with the typical clinical characteristics and identified a novel missense mutation p.I63N, which is localized in the forkhead domain and alters an evolutionarily conserved amino acid. Up to now, more than 40% of intragenic mutations of *FOXL2* affect the forkhead domain and can cause either type of BPES (12, 17). These strongly suggest that the novel mutation p.I63N in the *FOXL2* gene may be pathogenic and thus responsible for the pathogenesis of our BPES patients.

Besides expression in the developing eyelids, the transcription factor FOXL2 is an important regulator implicated in ovarian development and maintenance (19). Unlike the severe ovarian phenotype in *Foxl2* knockout mice, type I BPES patients caused by the heterozygous mutations usually undergo complete follicle development but early depletion of the

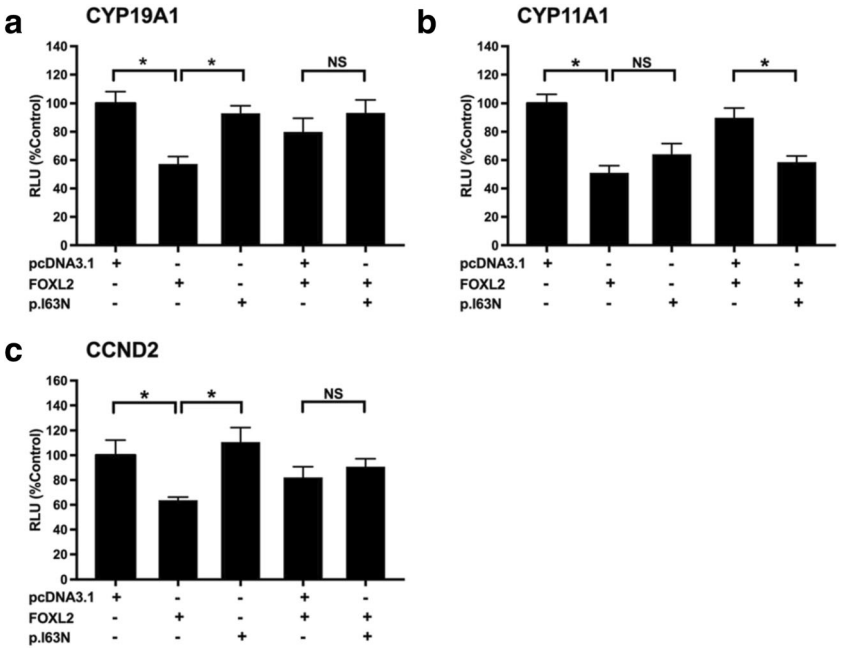

Fig. 2 Transcriptional activity of p.I63N mutant protein. Co-transfection of empty (pcDNA3.1), wild-type, or mutant *FOXL2* expression vectors and *CYP19A1* (A), *CYP11A1* (B), or *CCND2* (C) promoter reporter was performed in HEK293 cells. Results are expressed as a percentage of control activity (%Control). The potential dominant-negative effect of the p.I63N mutant was assessed by co-transfecting wild-type expression vector with empty or mutant vector (1:1) in HEK293 cells. *$P < 0.05$. *RLU*, relative light units

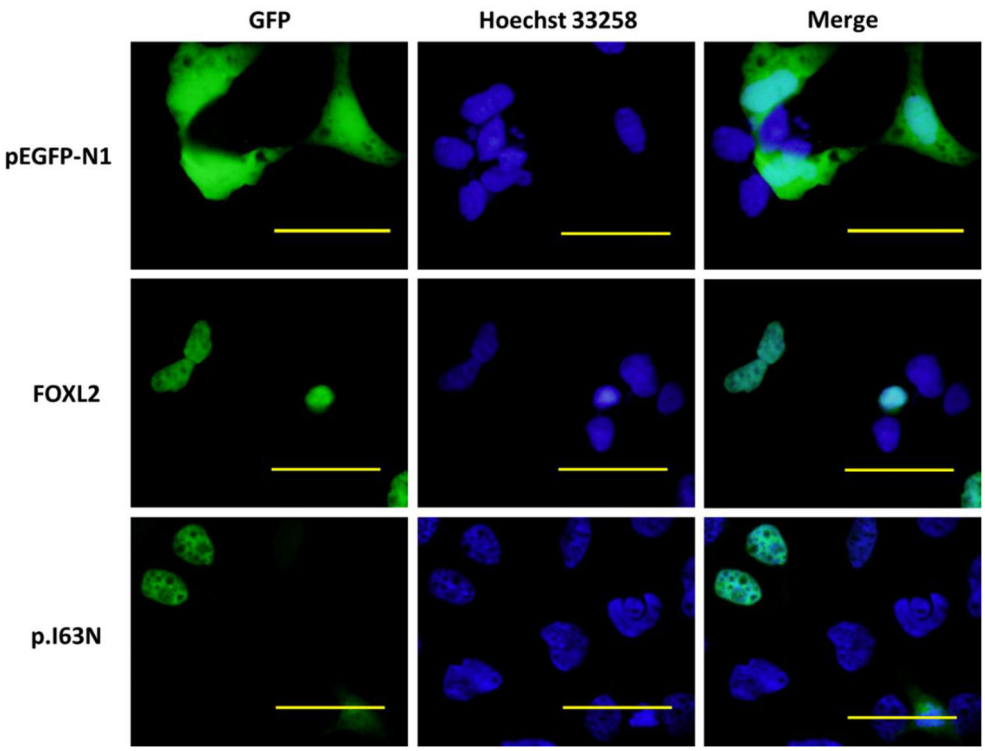

Fig. 3 Expression and localization of p.I63N mutant protein. HEK293 cells were transiently transfected with empty vector (pEGFP-N1), wild-type, or mutant *FOXL2* expression vector. The expression and cellular localization of the FOXL2 protein was indicated by GFP (green). The nuclei were counterstained with Hoechst 33258 (blue). Scale bars = 50 μm

follicle pool and subsequent ovarian failure (20). However, our BPES patients were infertile but did not exhibit POF till now, which might represent a new phenotype. It is also possible that the two patients are still younger than 40 years old. It has been revealed that, besides target genes in eyelid development, the mutations identified in type I BPES also disrupted crucial ovarian targets to account for the phenotype of ovarian dysfunction (17). In this study, we further illustrated whether the similar mechanism exited in our patients.

In ovary, FOXL2 affects granulosa cell proliferation, differentiation, and steroidogenesis by regulating the transcription of *STAR*, *CYP11A1*, *CYP19A1*, *CCND2*, and *AMH* genes, and the FOXL2 mutants result in dysregulated expression of these target genes, leading to ovarian dysfunction and depletion of follicles in type I BPES (10, 21, 22). Mutations in the *FOXL2* gene have also been identified in non-syndromic POF (23, 24). In our study, the FOXL2 mutation p.I63N abolished its transcriptional repression on *CYP19A1* and *CCND2* promoters. The upregulated expression of aromatase, which is encoded by *CYP19A1* and CCND2 protein, could contribute to premature estrogen synthesis and proliferation, respectively, ultimately leading to abnormal folliculogenesis. However, this mutant did not change the inhibitory effect on *CYP11A1* promoter indicating that the effects of this variant was promoter dependent and loss of function of this mutant was not complete toward the target genes in the ovary. Furthermore, unlike some mutations reported in previous studies (11, 25), our results showed that wild-type FOXL2's activity as a transcriptional repressor of the *CYP19A1* and *CCND2* promoters was equivalent when co-expressed with mutant *FOXL2*, which confirmed that mutant *FOXL2* did not exert a dominant-negative effect and suggested that haploinsufficiency was the pathogenic mechanism. All these may explain the relative mild ovarian dysfunction only manifesting infertility but without POF in these two BPES patients. Our results, together with previous reports (17, 18), suggest high heterogeneity in both genotype and phenotype in BPES patients.

Subcellular localization and aggregation pattern of the mutant FOXL2 proteins was found to be highly variable, and many missense mutations, including p.I63T and p.I63G, in forkhead domain led to subcellular mislocalization and/or protein aggregation (26, 27). It has been reported that protein mislocalization and aggregation negatively affect transcriptional activity of FOXL2 and may be considered as a predictor of ovarian dysfunction (17). While the mutation p.I63T was reported to derive from a BPES family (26, 28), the mutation p.I63N identified in this study did not change the nuclear localization and diffused distribution. But, the FOXL2 mutant p.I63N did abrogate the transcriptional repression of ovarian target genes including *CYP19A1* and *CCND2*, indicating that this mutation may affect the DNA-binding affinity of FOXL2 and also impair ovarian function without altering the subcellular localization.

In addition, it has been uncovered that FOXL2 could maintain the identity of granulosa cells by antagonizing expression of testis-specific genes (29, 30) and prevent oxidative damage by promoting DNA repair (31, 32), both of which also play important roles in ovarian function. The impairment of p.I63N

mutation on these functions of FOXL2 may also be involved in the occurrence of infertility in our BPES patients.

In conclusion, we identified a novel *FOXL2* mutation pI63N in a Chinese family suffering from BPES and infertility. Functional studies indicated that the mutant protein affected transcriptional repression on *CYP19A1* and *CCND2* while did not alter protein localization. The present study amplified the spectrum of *FOXL2* mutations and also revealed the possible pathogenesis of infertility at the molecular level in the BPES patients.

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Compliance with ethical standards

Declaration The authors have no competing interests.

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