

A Novel FOXL2 Missense Mutation, c.1068G>C, In Chinese Families with Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome Type II

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Abstract

Background Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) is a hereditary disease caused by a mutation in the forkhead box L2 (FOXL2) gene. Female patients suffering from premature ovarian failure (POF) were classified as type I, and others were classified as type II. We aimed to clarify a novel FOXL2 indel mutation in Chinese families and to predict the POF risk in the affected patient.

Methods Three generations of one Chinese family with BPES were enrolled in this study. Blood samples from patients of this family were collected and then analysed by whole-exome sequencing. Confocal microscopy was performed to observe the subcellular location. Transactivation studies were performed with real-time PCR.

Results This novel mutation (c.1068G>C) is located in the downstream of DNA-binding forkhead(FHD) domain, and the mutant protein could also exhibit transactivation capacity of StAR , a key regulator of POF. Conclusively, we discovered a novel FOXL2 mutation and predicted that female patient in this family should be classified as type II BPES.

Conclusions Our study revealed a novel missense mutation (c.1068G>C) and expanded the spectrum of FOXL2 gene mutations. Although we were not able to determine the classification from clinical manifestation, we discovered the patient developed type II BPES through subcellular distribution and transactivation analysis.

Background

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; OMIM #110100) is clinically manifested in the form of eyelid malformation caused by autosomal dominant inheritance, and its incidence is approximately 1/50000[1]. The characterization of this disease includes narrow horizontal palpebral fissures, congenital ptosis, telecanthus and epicanthus inversus[2]. BPES has been divided into two categories according to whether the absence of infertility in female patients is caused by premature ovarian failure (POF): type II lacks POF compared with type I[3]. Currently, a mutation in the forkhead box L2 (FOXL2) gene (OMIM #605597) is thought to be the main reason for both types of BPES[4].

FOXL2 is a 2.7-kb gene with a single exon located at chromosome 3q23. *FOXL2* encodes 376 amino acids, including a 100-amino acid DNA-binding forkhead domain (FHD) and a poly-Ala tract. FOXL2 is an important transcription factor expressed in ovary granulosa cells and the mesenchyme of the developing eyelid. Thus, mutant *FOXL2* should be responsible for aberrant ovaries, embryogenesis and eyelids[5]. Some reports have indicated that steroid metabolism and reactive oxygen species (ROS) detoxification are regulated by the *FOXL2* gene[6, 7]. To date, studies have revealed that some diseases, such as polycystic ovary syndrome (PCOS), adult-type ovarian granulosa cell tumours (GCTs) and lacrimal gland underdevelopment, are caused by aberrant *FOXL2*[8, 9].

Until now, intragenic mutations, such as frameshift mutations (most frequent), in-frame mutations, nonsense mutations and missense mutations, have been responsible for 71% of BPES cases[10]. A previous report claimed that type I was caused by a truncation before poly-alanine, whereas type II was often caused by poly-Ala expansion mutations[11]. However, the definite relationship between BPES and such mutations needs to be further investigated. A recent report provided a better method to identify whether a mutation will cause POF through a transactivation reporter system, such as FLRE-luc and SIRT1-luc[12]. Steroidogenic acute regulatory protein (StAR) is an essential factor involved in the follicular differentiation of granulosa cells and the maturation of ovarian antral follicles[13]. Normally, the function of *StAR* is repressed by FOXL2 binding to its promoter, and any mutation that impacts this connection will give rise to POF[14, 15].

Due to the patient inherited BPES syndrome from his father. We were not able to determine the classification from clinical manifestation. Here, we clarified a novel *FOXL2* mutation, c.1068G>C, from a Chinese family with BPES. In addition, this mutation was in the downstream of FHD domain and didn't disturb subcellular location and transactivation ability, which indicated the affected patient developed type II BPES with limited POF risk.

Methods

Patients

The Shanghai Ninth People's Hospital of Shanghai Jiaotong University School of Medicine diagnosed a family with BPES. BPES in this family could not be defined because the female patient was a prepubescent girl. Detailed clinical examinations carried out by ophthalmologists were consistent with the diagnostic criteria (blepharophimosis, ptosis, epicanthus, and telecanthus) (*Table 1*). To record the features of the patients, we took images before surgery. The proband of the family (III:2), an 8-year-old girl, acquired the pathogenic gene from her father. All affected patients presented with typical features of BPES, including small palpebral fissures, ptosis of the eyelids, epicanthus inversus, and telecanthus (*Figure 1A*). Informed consent for research was obtained from all participants or their guardians according to the tenets of the Declaration of Helsinki and Guidance of Sample Collection of Human Genetic Diseases through the Ministry of Public Health of China.

DNA extraction and sequencing

We collected blood samples from patients of this family and applied an Automatic Nucleic Acid Isolation System to obtain genomic DNA from the leukocytes of peripheral venous blood. We applied whole-exome sequencing to analyse genomic DNA, and the consequences were compared with the reference from the GenBank database. To analyse this novel mutation, we adopted three kinds of overlapping primers to amplify the *FOXL2* coding region as previous described[16]. The PCR amplification conditions were as follows: 95°C for 3 min, 40 cycles at 94°C for 60 s, 65°C for 30 s, and 72°C for 30 s; and a final elongation

step at 72°C for 5 min. The mutation analysed by Finch TV software and the final consequences were compared to the reference sequence from the GenBank database.

Plasmid construction

Complementary DNA (cDNA) encoding wild-type FOXL2 and mutant FOXL2 were cloned with PCR and then inserted into the pcDNA3.1 and EGFP-N1 plasmids for constructing pcDNA3.1-FOXL2-wild-type and N1-FOXL2-EGFP-wild-type plasmids, respectively. All plasmids were isolated and purified with anion exchange columns (QIAGEN, Hilden, Germany). Plasmids were sequenced to confirm that there were no additional mutations.

Subcellular location of FOXL2-WT and FOXL2-MT

293T cells were seeded into 6 cm dishes and cultured to 50–60% confluence. Then, EGFP-FOXL2-wild-type and EGFP-FOXL2-mutant plasmids were transfected into 293T cells with the help of 3 µl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 72 hours, confocal laser scanning microscopy was used to recognize the location of these two proteins.

Real-time PCR

Quantitative SYBR Green real-time (RT) PCR was used to evaluate the expression of endogenous StAR. Chinese hamster ovary cells (CHO) were cultured in 6 cm dishes and transfected with 10 µg of pcDNA3.1-FOXL2-wild-type/mutant/empty plasmid for 72 hours. Following the manufacturer's instructions, TRIzol (Invitrogen) was used to extract total mRNA from cells. Reverse transcription (RT)-PCR was performed with 2 µg of RNA using Super script II (Invitrogen). The housekeeping gene GAPDH was used as an endogenous control.

Results

BPES patients with a novel mutation

We diagnosed 2 patients in a three-generation, 13-member Chinese family with BPES, as shown in *Figure 1A*. Patients were carefully examined by an ophthalmologist based on the following criteria: blepharophimosis, ptosis, epicanthus inversus and telecanthus (*Table 1, Figure 1B*). Whole-genome sequencing analysis of genomic DNA extracted from patients' peripheral blood leukocytes revealed a novel missense mutation in guanine deoxynucleotides replacing cytosine deoxynucleotides at the 650th base of the CDS of *FOXL2* (*Figure 1C*). This novel missense mutation caused an aberrant amino acid sequence in which serine (Ser) at the 217th amino acid position was replaced by cysteine (Cys), as shown

in *Figure 1D*. The mutation was located downstream of the forkhead domain, upstream of the poly-Ala domain (*Figure 2*).

Mutated protein is located in the nucleus.

To observe the subcellular localization of the FOXL2 mutant protein, we constructed wild-type and mutant *FOXL2* plasmids with an EGFP tag and transfected them into 293T cells. 293T cells are a suitable experimental option because 293T cells lack FOXL2 gene expression, thus eliminating endogenous interference. Here, we observed that both wild-type and mutant FOXL2 were distributed in the nucleus (*Figure 3, left panel*). Thus, this novel mutation, c.1068G>C, did not disturb FOXL2 function as a transcription factor. This mutation may have no impact on gene transactivation activity, because the subcellular localization of mutant FOXL2 was also diffusely distributed only in the nucleus (*Figure 3, right panel*).

Mutated FOXL2 also represses downstream *StAR*

A previous study revealed that FOXL2 strongly repressed the expression of *StAR* expression, a key regulator in the pathogenesis of POF, by binding to its promoter[11, 17]. To further confirm whether this novel mutation abolished its transactivation capacity on *StAR*, wild-type *FOXL2* and mutant *FOXL2* were transfected into Chinese hamster ovarian (CHO) cells (*Figure 4A*). Notably, *StAR* expression was silenced after transfecting wild-type or mutant *FOXL2* (*Figure 4B*). This result confirmed that this novel mutant *FOXL2* could also regulate *StAR* expression as a transcription factor.

Discussion

FOXL2 is an evolutionarily conserved forkhead transcription factor that affects reproductive system development, sex determination, metabolism and tumourigenesis and has been shown to be associated with multiple diseases, such as polycystic ovary syndrome (PCOS)[18], pathologic scar formation[19] and reproductive system tumours[20]. FOXL2 is highly expressed in the ovary and eyelids, and its mutation causes BPES[21]. In addition, FOXL2 could direct bind to the promoter of its target gene and repress its expression, such as *StAR* and *SIRT1*[22]. Because *StAR* and *SIRT1* play a crucial role in the functional maturation of ovarian antral follicles, mutated FOXL2 could not only result in the malformation of the eyelid but also infertility[23].

It has been reported that BPES type I is closely associated with the truncation of the FOXL2 protein before the poly-Ala tract, whereas BPES type II is caused by poly-Ala expansions[24, 25]. However, the situation is not exactly as it is described, and sometimes variation may occur. Some patients with type II BPES harbour a truncation in the FOXL2 protein before the poly-Ala tract, and type I BPES is caused by poly-Ala expansions[26]. It has also been reported that the same mutation in one family causes different types of

BPES[26]. This phenotypic variability may result from the differences in the genetic background of each individual.

In our study, through whole-exome sequencing, we found a novel FOXL2 missense mutation (c.1068G>C) located in the downstream of the DNA-binding forkhead domain. It should be noted that the two types of BPES were classified according to whether infertility was absent in female patients[27]. Here, we could not distinguish the clinical type of BPES in this patient from clinical information, because of the paternal inheritance. Here, we observed the subcellular distribution and tested the transactivation capacity of FOXL2 and predicted the limited risk of POF in the affected girl.

Conclusion

Taken together, our results revealed a novel missense mutation (c.1068G>C) in favour of a BPES family. Although we were not able to determine the BPES classification from clinical manifestation, we revealed the affected patient developed type II BPES through subcellular distribution and transactivation analysis. Our study expands the spectrum of known FOXL2 gene mutations and provided an alternative method for the prediction of POF risk in BPES patients.

List Of Abbreviations

BPES: blepharophimosis-ptosis-epicanthus inversus syndrome

FOXL2: forkhead box L2

POF: premature ovarian failure

StAR: steroidogenic acute regulatory protein

FHD: forkhead domain

ROS: reactive oxygen species

PCOS: polycystic ovary syndrome

GCT: granulosa cell tumour

Ser: serine

Cys: cysteine

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of the Ninth People's Hospital of Shanghai JiaoTong University School of Medicine. For all involved individuals, only non-identifiable information was presented. Written, informed consent was obtained for all involved patients. Informed consent was obtained from all patients or their guardians at the follow-up visit.

Consent for publication

Written, informed consent was obtained for all involved patients. A form letter containing the carrier screening agreement for children younger than 18 years old was signed by their parents.

Availability of data and materials

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

Competing interests

The author declare that they have no competing interests.

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Authors' contributions

In this report, SYW and FLH designed and performed the experiments and drafted the manuscript; RBJ were responsible for sample collection and data analysis; XS, RBJ and SFG discussed, revised and approved the manuscript. All authors approved this manuscript.

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Tables

Table 1. Clinical features of the Chinese family with BPES.

Patient	Age (years)	IICD (mm)	IPFH (mm)		HPFL (mm)		Levator function (mm)	
			RE	LE	RE	LE	RE	LE
II -1	36	34	3	2	24	23	2	2
III -2	8	33	3	3	22	22	2	2

Abbreviations: IPFH: vertical interpalpebral fissure height; HPFL: horizontal palpebral fissure length; IICD: inner intercanthal distance; LE: left eye; RE: right eye.

Figures

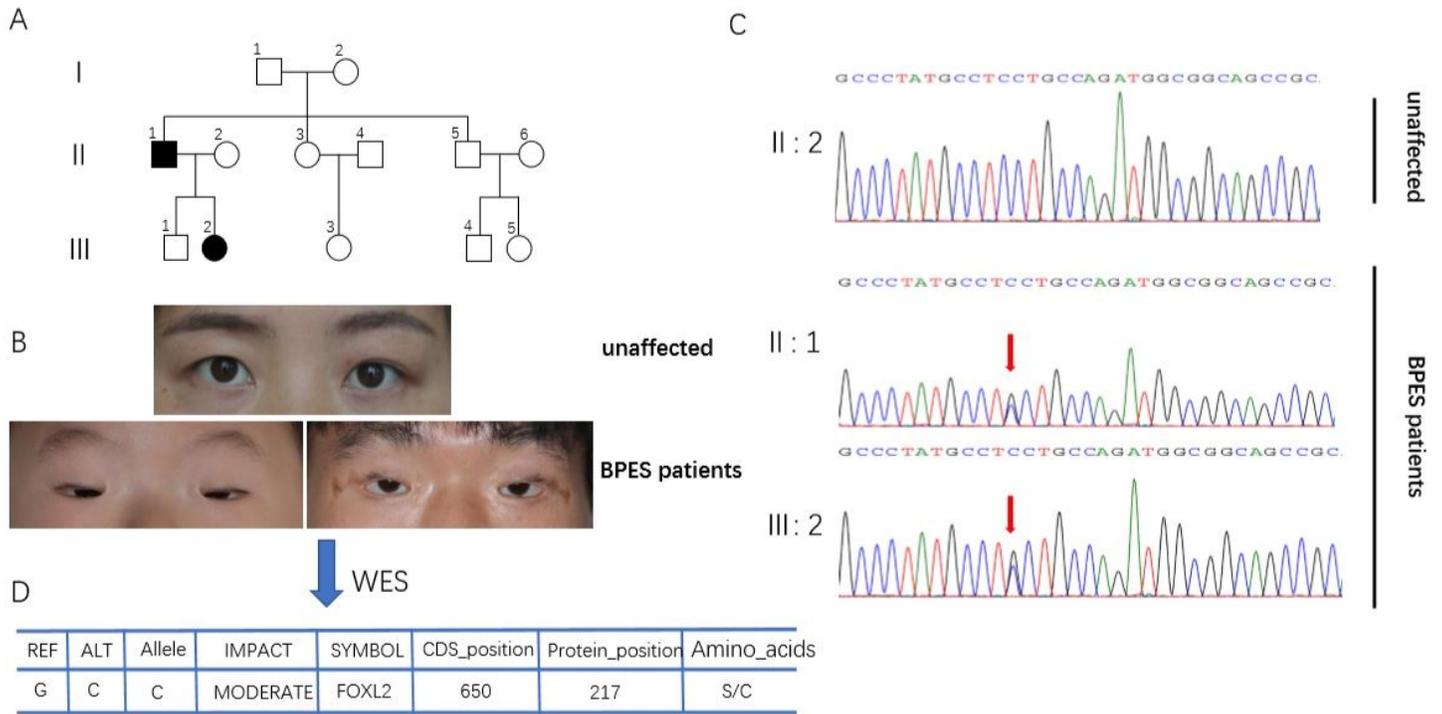
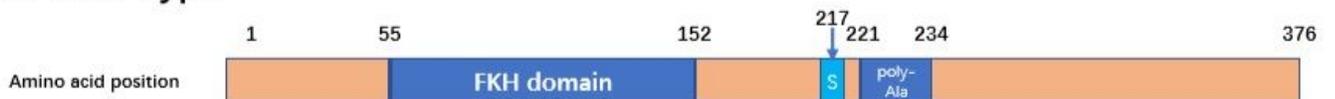


Figure 1

Facial features and genomic analysis of the FOXL2 gene. (A) Three-generation BPES pedigree. (B) Facial features of the patients before surgery. (C) Whole-exome sequencing revealed a novel FOXL2 missense mutation [c.1068G>C] in affected members of this family with BPES. (D) Aberrant FOXL2 amino acid sequence in which serine (Ser) at the 217th amino acid position was replaced by cysteine (Cys).

FOXL2 Wild-type



FOXL2 Mutant type

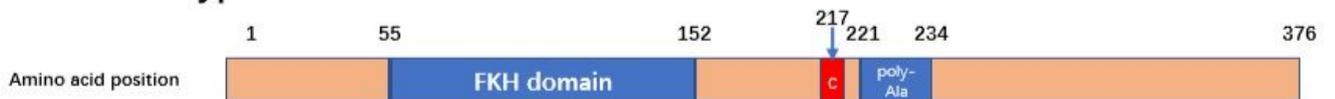


Figure 2

A novel FOXL2 missense mutation [c.1068G>C] results in serine (Ser) at the 217th amino acid position being replaced by cysteine (Cys).

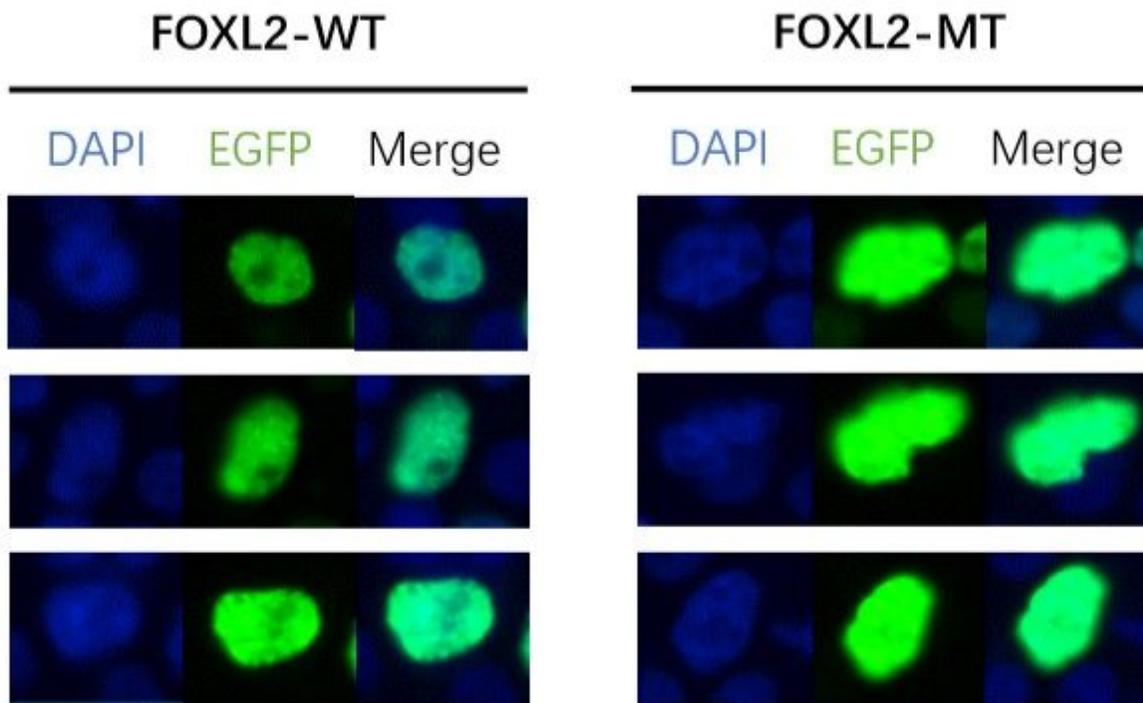


Figure 3

Intracellular localization of WT and MT FOXL2 proteins. The left panel is a representative image showing nuclear staining with DAPI. The middle panel shows the subcellular localization of FOXL2 as a fusion protein with EGFP. The right panel is a merged image of the previous two images. Both wild-type and mutant FOXL2 proteins are located in the nucleus.

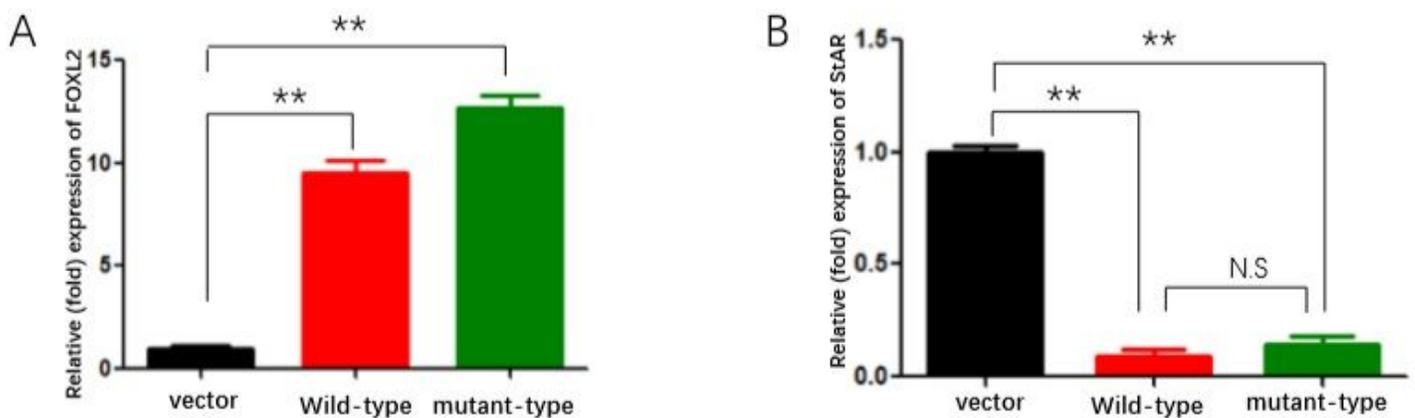


Figure 4

FOXL2 transactivation capacity analysis. (A) Relative mRNA expression of FOXL2 in CHO cells transfected with wild-type or mutant FOXL2. (B) RT-PCR shows that both wild-type and mutant FOXL2

repress StAR expression.