

Identification of the forkhead transcriptional factor 2 (*FOXL2*) gene mutations in four Chinese families with blepharophimosis syndrome

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Purpose: To determine the genetic origin of disease in four Chinese families with blepharophimosis syndrome.

Methods: Four Han Chinese families with blepharophimosis syndrome were ascertained and patients underwent complete physical and ophthalmic examinations. Blood samples were collected and genomic DNA was extracted. Sequence analysis of the forkhead transcriptional factor 2 (*FOXL2*) gene was performed by direct sequencing and mutations were analyzed.

Results: Three mutations in *FOXL2* were found in four families, including c.672_701dup30 (p.Ala224_Ala234dup10), c.313C>A (p.N105H), and c.430G>T (p.R144W). The c.672_701dup30 (p.Ala224_Ala234dup10) mutation was reported previously and predicted to result in expansions of the polyalanine tract. The mutations of c.313C>A (p.N105H) and c.430G>T (p.R144W) are two novel missense mutations.

Conclusions: Our study further supports the view that the expansion of the polyalanine tract is the hotspot of mutations within *FOXL2*. The two novel missense mutations detected in this study will expand the mutation spectrum of the *FOXL2* gene and contribute to the research on the molecular pathogenesis of *FOXL2*.

Blepharophimosis syndrome (BPES, MIM #110100) is a rare autosomal dominant inheritable disorder with an estimated incidence of 1 in 50,000 births [1]. It is a complex eyelid disease characterized by blepharophimosis, ptosis, epicanthus inversus, and telecanthus. Other ocular abnormalities with BPES are strabismus, refractive error, and amblyopia in one or both eyes [2]. There are two forms of BPES distinguished by the eyelid defects with (type I) or without (type II) premature ovarian failure [3]. However, both types of BPES are caused by mutations of the forkhead transcriptional factor 2 (*FOXL2*) gene spanning approximately 2.7 kb on chromosome 3q23.

FOXL2 (OMIM 605597) is a member of the superfamily of Forkhead Box (Fox) transcription factors. It is a single-exon gene and encodes a protein with 376 residues containing a 100 amino acid DNA-binding forkhead domain and a polyalanine (poly-Ala) tract that is highly conserved in mammals. Currently, more than 100 different mutations have been identified in *FOXL2*, including frameshifts,

insertions, nonsense mutations, and missense mutations [4,5]. Although some authors have found that mutations resulting in a predicted truncated protein before the poly-Ala tract are associated with BPES type I, whereas mutations resulting in an extended protein might lead to BPES type II, no clear genotype-phenotype correlation has been confirmed between mutations and BPES types because the genetic and clinical heterogeneity are found in and between families with BPES [6,7].

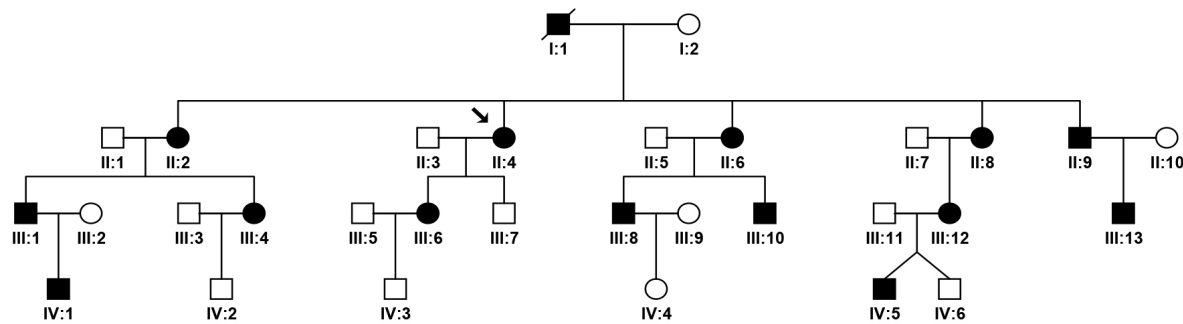
Here, we report three mutations identified in the *FOXL2* gene from four Chinese families with BPES. Two of the mutations are missense, and one of them is indel.

METHODS

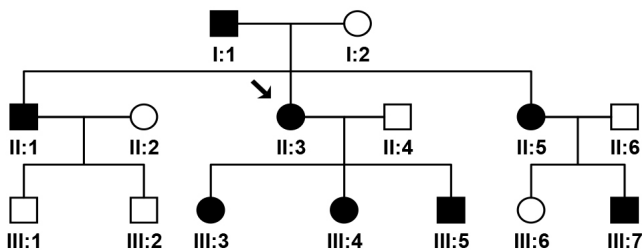
Patient ascertainment: Four Han Chinese families with BPES (Figure 1) were recruited through the Ophthalmic Genetics Clinic of the Tianjin Eye Hospital, Tianjin, China. Patients with BPES were diagnosed depending on the following clinical criteria: blepharophimosis, ptosis, epicanthus inversus, and telecanthus. Premature ovarian failure was defined as cessation of menses for a duration of ≥ 6 months before the

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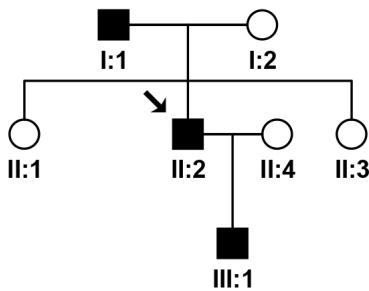
A. c.672_701dup30 (p.Ala224_Ala234dup10)



B. c.672_701dup30 (p.Ala224_Ala234dup10)



C. c.313C>A (p.N105H)



D. c.430G>T (p.R144W)

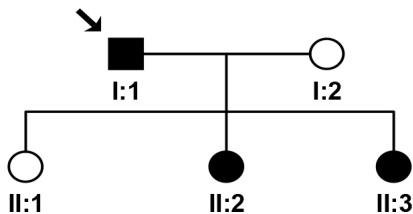


Figure 1. Pedigrees and respective forkhead transcriptional factor 2 (*FOXL2*) mutations of patients with blepharophimosis syndrome (BPES). Squares and circles indicate males and females, respectively, and the darkened symbols represent the affected members. The patients with the arrow above are the probands.

age of 40 and a concentration of follicle-stimulating hormone of >40 IU/l.

After informed consent was received, 3 ml blood samples were taken from the blood vessels of 27 affected and 21 unaffected individuals from the four unrelated families, and DNA was extracted from blood lymphocytes according

to the standard methods of protocol (Roche Biochemical, Inc. Penzberg, Germany). This study obtained institutional review board (IRB) approval from the Tianjin Eye Hospital and conformed to the tenets of the Declaration of Helsinki.

Mutation analysis: Amplification of the genomic fragments encompassing *FOXL2* coding regions (NCBI human genome

TABLE 1. THE PRIMER PAIRS FOR AMPLIFICATION OF THE FORKHEAD TRANSCRIPTIONAL FACTOR 2 (FOXL2).

Name	Forward	Reverse	Size (bp)
Frg-1	TCCGCAGTCTCCAGAAGTTT	GACAGCGTGAGCCTCTTCTC	354
Frg-2	GCACAGTCAAGGAGCCAGA	GCCCTTCTCGAACATGTCTT	359
Frg-3	CCCGTTCTACGAGAAGAATAAG	ATCTGGCAGGAGGCATAGG	396
Frg-4	ACATGTTTCGAGAAGGGCAAC	CCAGGCCATTGTACGAGTTC	426
Frg-5	CCTATGCCTCCTGCCAGAT	GTCGTGGTCCCAGTAAGAGC	452
Frg-6	CCGGCGTAGTGAACCTCGTA	AAAGCGAAAAAGCACAGAGG	486

build 35.1, [NC_000003](#) for gDNA, [NM_023067](#) for messenger RNA, and [NP_075555](#) for protein) was performed by PCR using the primers in Table 1. PCR was carried in 20 µl of standard PCR buffer containing 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 U of Taq polymerase, and 50 ng of DNA. The amplification program was an initial 2 min denaturation at 98 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and a final 7 min extension step at 72 °C. The PCR products were separated on a 2% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

DNA sequencing analysis was performed using the BigDye Terminator Cycle Sequencing V3.1 kit on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequencing results were assembled and analyzed with the Seqman program of DNASTAR software (DNASTAR Inc., Madison, WI). Each heterozygous mutant was further validated with bidirectional sequencing in all relatives of the

affected families and 100 normal healthy controls. Cloning sequencing was performed for the validation of multiple nucleotides insertions. A multiple sequence alignment was performed using the Clustal W algorithm in the software package. Mutation naming followed the nomenclature recommended by the Human Genomic Variation Society.

RESULTS

All patients in the four families were found to have typical features of BPES, including small palpebral fissures, ptosis of the eyelids, and epicanthus inversus (Figure 2). The clinical forms of families A and B were classified as BPES type II because the affected females in these two families did not have fertility problems. However, we were unable to evaluate the BPES types for families C and D because there were no female patients in family C, while the two affected females in family D were of pubertal age and had normal hormonal measurements and menstrual cycle (data not shown).

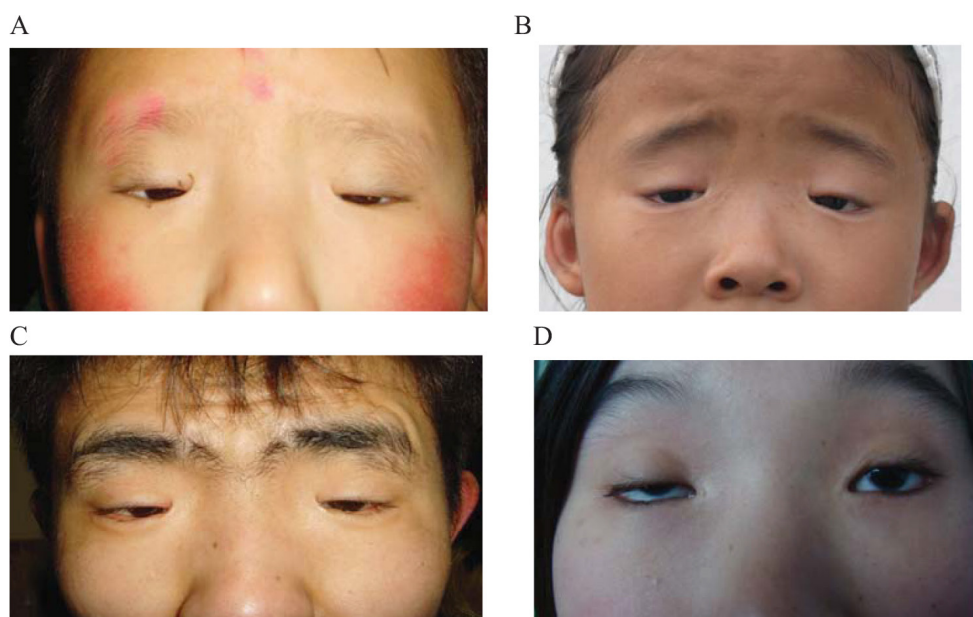


Figure 2. Characteristic clinical features of the patients with blepharophimosis syndrome (BPES). **A, B, C, D:** The photos represent affected individuals from the four families with BPES. All patients have small palpebral fissures, ptosis of the eyelids, and epicanthus inversus.

TABLE 2. SEQUENCE ANALYSIS OF FOUR SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN TWO PROBANDS FROM TWO FAMILIES CARRYING WITH C.672_701DUP MUTATION.

SNP	Location	Family A	Family B
rs56799713	138660471	C/C	C/A
rs1482976	138661115	T/T	C/T
rs12630172	138662552	G/G	G/T
rs2291252	138662983	G/G	G/G

in both family A and B. It is a frameshift mutation within the coding region of the poly-Ala tract and was predicated to result in poly-Ala expansions. To examine the possibility of a common origin between family A and B, four single-nucleotide polymorphisms ([rs56799713](#), [rs1482976](#), [rs12630172](#), and [rs2291252](#)) harboring the *FOXL2* locus were genotyped in the probands of the families of A and B (Table 2). The haplotypes of these two probands were quite distinct. This suggested that the same mutation, c.672_701dup30 (p.Ala224_Ala234dup10), occurred independently in these two families rather than that they had descended from a common ancestor.

The heterozygous mutations of c.313C>A and c.430G>T were detected in family C and D, respectively. The C to T change at nucleotide position 313 would result in an asparagine at codon 105 substituted by a histidine (N105H), whereas the C to T change at the nucleotide position 430 would be

predicted to result in an arginine at codon 144 replaced by a tryptophan residue (R144W).

The multiple sequence alignment of the *FOXL2* protein shows that N105H and R144W were conserved among human, Pongo (chimpanzee), mouse, cow, chicken and zebrafish (Figure 4). The N105H and R144W amino acid changes produce PROVEAN scores of -4.943 and -6.766, respectively, as predicted by the [PROVEAN](#) tool. Both of them produce a position-specific independent counts (PSIC) profile score of 1.0 calculated by the [POLYPHEN2](#) program. These values indicate that the amino acid substitutions at N105 and R144 are likely to have a deleterious effect on the protein. These results suggest that the mutations of c.313C>A (p. N105H) and c.430G>T (p.R144W) are novel mutations in the *FOXL2* gene.

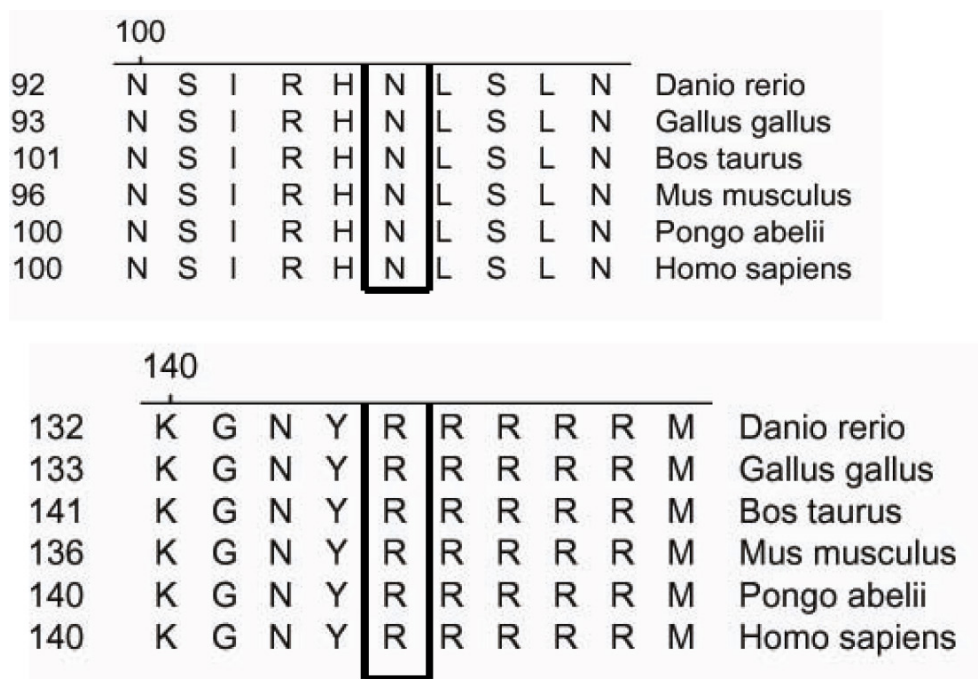


Figure 4. Sequence alignment of the forkhead transcriptional factor 2 (*FOXL2*) gene product among the human, Pongo, mouse, cow, chicken and zebrafish. The N105 and R144 are conserved among all of these species.

DISCUSSION

As mentioned above, *FOXL2* is a member of the winged helix/forkhead transcription factor family, and is mainly expressed in the developing eyelid and ovarian follicular granulosa cells. It encodes a protein with 376 amino acid residues, which contains a 100 amino acid DNA-binding forkhead domain from amino acid position 52 to 152 and an alanine-rich domain from amino acid position 221 to 234 downstream of the forkhead domain. Currently, 46 missense mutations have been found in the *FOXL2* gene. Thirty-two of these are located in the forkhead domain [6-18], 1 in the alanine-rich domain, 10 in the region flanked by the forkhead and alanine-rich domains [9,12,17,19-22], and 3 downstream of the alanine-rich domain [6,8]. These missense mutations might lead to the BPES phenotype either by haploinsufficiency or through the dominant-negative effect. However, the exact molecular pathogenesis mechanism is not yet clear, nor can genetic and phenotype correlations be established for these missense mutations.

The missense mutation of N105H was located in the DNA-binding forkhead domain. It was caused by a C to A change at the nucleotide position 313. The mutation at N105 was reported previously by Crisponi et al. [23], and comprises a substitution of a serine (S) to an asparagine (N) due to an A to T change at the nucleotide position 314. The asparagine (N) at codon 105 is highly conserved among vertebrates and lower animals, and may provide key sites for N-linked glycosylation when occurring in an Asn-X-Ser/Thr form in the motif [24]. Because glycosylation is important for both protein structure and function, substitution of the asparagine either by a serine or a histidine would be deleterious to the structure and function of the protein.

The R144W mutation was also located in the DNA-binding forkhead domain. R144 is highly conserved in vertebrates and low animals, and localized at the first position in the arginine/lysine (RK)-rich region (R₁₄₄R₁₄₅R₁₄₆R₁₄₇R₁₄₈M₁₄₉K₁₅₀R₁₅₁) at the C-terminal of the forkhead domain. The RK-rich region is conserved in several forkhead domains and might function as a nuclear localization signal. Mutations in the RK-rich region have been demonstrated to lead to mislocalization of the proteins [25]. The missense mutation of R144W would affect the protein structure and function, as predicted by the PROVEAN tool and POLYPHEN2 program.

Previous studies showed that missense mutations inside or outside the forkhead domain could determine the expressivity of BPES. Mutations inside the forkhead domain might produce a more severe phenotype, while mutations outside it might produce a mild phenotype, except in an Indian BPES family carrying a missense mutation outside the forkhead

domain but with a severe phenotype [15,26]. In this study, BPES families carrying either the N105H or R144W mutation had a typical clinical BPES phenotype, which further supported the possibility that the affected BPES individuals with missense mutations inside the forkhead domain might have a severe or typical phenotype.

The c.672_701dup30 mutation was reported previously and localized in the alanine-rich domain. Thus far, the role of the alanine-rich domain in *FOXL2* has not yet been elucidated. It might be related to the function and structure of the protein because the number of alanine residues is strictly conserved among mammals. It was reported that the alanine-rich domain was a mutational “hotspot,” since the majority of the in-frame mutations occurred in this region and resulted in poly-Ala expansions. The possible mechanism for the in-frame changes in the poly-Ala tract of *FOXL2* is the DNA hairpin-induced polymerase slippage that leads to the poly-Ala expansions [7]. The poly-Ala expansions might lead to protein mislocalization and aggregation, which results mainly in BPES type II [27]. Our study further supports that the poly-Ala domain is a mutational hotspot for these two independent families with BPES type II carried the same mutation of c.672_701dup30 in this region.

In summary, we identified three mutations—c.672_701dup30 (p.Ala224_Ala234dup10), c.313C>A (p.N105H), and c.430G>T (p.R144W)—in the *FOXL2* gene in four Han Chinese families with BPES. The c.672_701dup30 was detected in the two families diagnosed as BPES type II, while the two missense mutations of c.313C>A and c.430G>T are novel mutations detected in the other two families with unknown types of BPES. Our data further support that poly-Ala expansions lead to BPES type II. For missense mutations, no predictions can yet be made because of a lack of sufficient information from families. The two novel missense mutations would expand the mutation spectrum of the *FOXL2* gene and contribute to the study of the molecular pathogenesis of BPES.

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