**FOXL2** mutations in Chinese patients with blepharophimosis-ptosis-epicanthus inversus syndrome

Juan Wang,1 Jinling Liu,2 Qingjiong Zhang1

(The first two authors contributed equally to this publication)

1State Key Laboratory of Ophthalmology, and 2Eye Hospital, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China

**Purpose:** Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) is an autosomal dominant disorder where eyelid malformation associated with (type I) or without (type II) premature ovarian failure (POF). It is ascribed to mutations in the forkhead transcriptional factor 2 (**FOXL2**) gene. The purpose of this study is to identify mutations in **FOXL2** of Chinese patients with BPES.

**Methods:** Genomic DNA was prepared from leucocytes of peripheral venous blood. The coding regions and nearby intron sequences of **FOXL2** were analyzed by cycle and cloning sequencing.

**Results:** Four mutations in **FOXL2** were identified in six families, including c.241T>C, c.650C>G, c.804dupC, and c.672-701dup. Of the four, the c.241T>C and c.650C>G were novel and would result in missense changes of the encoded proteins, i.e., p.Tyr81His and p.Ser217Cys, respectively. The c.672-701dup (p.Ala224_Ala234dup) was detected in three families, indicating a mutation hotspot. The c.804dupC (p.Gly269ArgfsX265) mutation was found in one family.

**Conclusions:** Our results expand the spectrum of **FOXL2** mutations and confirm the mutation hotspot in **FOXL2**.

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES, OMIM 110100) is a rare autosomal dominant disease with a prevalence of about 1 in 50,000 [1]. Clinically, BPES has been divided into two subsets depending on the association of ocular malformation with (type I) or without (type II) premature ovarian failure (POF) [2]. Genetically, however, both types are caused by mutations in **FOXL2**, and a genotype-phenotype correlation has been described in some cases [3,4].

The human **FOXL2** gene (OMIM 605597), located at 3q23, is a member of winged/forkhead transcription factor gene family [5]. This single-exon gene codes a protein with 376 residues, which consists of a DNA-binding forkhead domain (residues 52-152) and a polyalanine domain (residues 221-234) [3,6,7]. A number of mutations in **FOXL2** have been identified [8], including six novel mutations in the Chinese population [9-11].

Here, we report four mutations identified in six Chinese families with BPES. Two novel missense mutations were associated with BPES type II.

**METHODS**

**Patients:** Thirteen probands with BPES from unrelated families were collected from the Zhongshan Ophthalmic Center. Informed consent conforming to the tenets of the Declaration of Helsinki and following the Guidance of Sample Collection of Human Genetic Diseases (863-Plan) by the Ministry of Public Health of China was obtained from all participated individuals or their guardians prior to the study. The diagnosis of BPES was based on criteria previously established [12] with exclusion of microphthalmia.

**Mutation Analysis:** Genomic DNA was prepared from leucocytes of peripheral venous blood [13]. Amplification of the genomic fragments encompassing **FOXL2** coding regions (NCBI human genome build 35.1, NC_000003 for gDNA, NM_023067 for mRNA, and NP_075555 for protein) was carried out by PCR using primers as follows: AF: 5’-CAG CGC CTT GAG AGA ACG GTG G-3’, AR: 5’-GCC CGC CTC TCC ACT GAC A-3’, CR: 5’-GCT GGC GGC GCC TGC GAA GAC A-3’, BR: 5’-GCC CGC GTC GAT GTG T-3’, BF: 5’-CGC GCC CGC TGT GGT CAA G-3’, CF: 5’-GCT GCC GCC GGC GGC GGC CTC GTC-3’. The sizes of the amplified DNA fragments are 545 bp, 517 bp, and 500 bp, respectively.

PCR amplification was carried out initially at 95 °C for 8 min, followed by 5 cycles at 94 °C for 40 s, at 68 °C for 40 s, at 72 °C for 40 s, then 5 cycles at 94 °C for 40 s, at 66 °C for 40 s, at 72 °C for 40 s, and a further 30 cycles at 94 °C for 40 s, at 64 °C for 40 s, at 72 °C for 40 s, and finally an elongation step at 72 °C for 5 min. Due to the high GC-rich nature of **FOXL2**, an additional 10% dimethylsulfoxide and 10% glycerol were added to the PCR mixture in order to successfully amplify the genomic fragments.

Direct sequencing of the PCR products was performed with an ABI BigDye Terminator Cycle Sequencing Kit v3.1 (ABI Applied Biosystem, Foster City, CA), using an ABI 3100 Genetic Analyzer. Sequencing results from patients as well as...
the FOXL2 consensus sequences from the NCBI Human Genome Database (NC_000003) were imported into the SeqManII program of the Lasergene package (DNAStar Inc., Madison, WI) and then aligned to identify variations. Each mutation was confirmed by bidirectional sequencing. Mutation description followed the nomenclature recommended by the Human Genomic Variation Society (HGVS).

Any variation detected in FOXL2 was further evaluated in available family members as well as in 100 normal controls by heteroduplex-SSCP analysis as described previously [14]. Two additional pairs of primers were used for heteroduplex-SSCP analysis. The sequence of these primers were: DF: 5’-CCG TAA GCG GAC TCG TGC-3’, DR: 5’- AGT AGT TGC CCT TGC GCT C-3’, EF: 5’- CGC ACT TCC AGC CCG GCA A-3’, and ER: 5’- TGT GTA CGG CCC GTA CGA-3’.

In addition, one variation of insertions with multiple nucleotides that was found in three families was further analyzed by cloning sequencing. PCR products harboring this mutation were subcloned to pMD18-T Simple Vector (TaKaRa BIO, Japan) according to the manufacture’s instructions. Clones with the mutant allele as well as the normal allele were selected by using heteroduplex-SSCP analysis. Sequence of the cloned fragment was identified by cycle sequencing as described above. Mutations were confirmed by sequencing three positive clones from each family. One mutation, c.241T>C, was further analyzed by PCR-RFLP analysis since the mutation creates a new FOKI site.

RESULTS
All patients demonstrated typical features of BPES, including small palpebral fissures, ptosis of the eyelids, and epicanthus inversus (Figure 1). Upon complete sequencing analysis of FOXL2 for 13 probands with BPES, four heterozygous mutations were found in six probands, including c.241T>C, c.650C>G, c.804dupC, and c.672_701dup (Figure 2; Table 1). Of the four, c.241T>C and c.650C>G are novel. All four heterozygous mutations were further detected by heteroduplex-SSCP analysis, and one (c.241T>C) was further detected by FOKI digestion (Figure 3). These mutations were also present in affected patients from corresponding families but neither in unaffected individuals nor in 100 controls.

The c.241T>C (p.Tyr81His) mutation results in substitution of a charge-free tyrosine with a charge-positive basic hydrophilic histidine within the forkhead domain. The c.650C>G (p.Ser217Cys) mutation is located immediately upstream of the polyalanine domain. The tyrosine at position 81 and the serine at position 217 are well conserved in FOXL2 by ClustalW analysis of 11 orthologs from related vertebrate species (Figure 4).
Table 1. *FOXL2* mutations detected in Chinese patients with BPES

| Family | DNA change | Mutation type | Location | Protein change | BPES      |
|--------|------------|---------------|----------|---------------|-----------|
| A      | c.241T>C   | Missense      | Forkhead | Tyr81His      | Type II   |
| B      | c.650C>G   | Missense      | Immediately upstream polyalanine | Ser217Cys | Type II   |
| C      | c.804dupC  | Insertion     | Downstream of polyalanine | Gly269ArgfsX265 | Unknown   |
| D      | c.672_701dup | Duplication | Polyalanine domain | Ala224_Ala234dup | Type II   |
| E      | c.672_701dup | Duplication | Polyalanine domain | Ala224_Ala234dup | Unknown   |
| F      | c.672_701dup | Duplication | Polyalanine domain | Ala224_Ala234dup | Unknown   |

Subtypes of BPES in families C, D, and F are unknown, as there were no female patients (families C and F) or the female patients were too young (family D, where the two female patients were only 4 or 2 years old, respectively).

Figure 3. Pedigrees and heteroduplex-SSCP analysis. Pedigrees of the different families (A, B, C, D, E, and F) are shown. Black filled symbols indicated patients affected with BPES in each family. The “+/-” or “+/-” sign indicated individuals analyzed with normal sequences or heterozygous mutation in *FOXL2*, respectively. The “M” under each lane indicated mutation, the “N” represented normal individuals, and “N+M” represented a mixture of PCR products resulted from normal and mutant clones. In addition, results of FOKI digestion for family A were present at the bottom of Figure 1A. The c.241T>C mutation in family A creates an additional FOKI site. By FOKI digestion of the 545 bp PCR product, normal allele yielded two fragments (340 bp and 205 bp), but the mutant allele yielded three fragments (275 bp, 205 bp, and 65 bp). Patients with the heterozygous c.241T>C mutation had four bands (only three bands were shown in the figure) as compared to normal individuals with two bands.
FOXL2 encodes a forkhead transcription factor containing a forkhead domain for DNA-binding and a polyalanine domain of uncertain function. Strong expression of FOXL2 has been found in eyelids [3,15], developing periorcular muscles, and surrounding tissues [16,17]. Of the four mutations identified in this study, the c.241T>C affected the forkhead domain, while the other three (c.650C>G, c.804dupC, and c.672_701dup) were located upstream, within, and downstream of the polyalanine domain, respectively.

Missense mutations in FOXL2 reported so far usually occurred at the forkhead domain [9,17-19], except two, such as c.650C>T in a Belgian family [4] and c.644A>G in a five-generation family from south-India [20]. The clinical subtypes of the patients with the c.650C>T and c.644A>G mutations were unknown. The novel c.650C>G (p. Ser217Cys) mutation identified in Chinese family B occurred at the same site as that found in the Belgian family, which is located immediately upstream of the polyalanine domain. The serine at position 217 is well conserved in 11 orthologs (Figure 4). It has been shown that mutations affecting the polyalanine domain induce extensive nuclear and cytoplasmic protein aggregation [21,22]. Missense changes have been suggested to act as null allele leading to BPES phenotype due to haploinsufficiency [4] or dominant-negative effect [20,23].

It has been suggested that FOXL2 mutations truncating the protein led to BPES type I while those extending the mutant protein were associated with type II [3,4]. However, intra- and inter-family phenotypic variations have been found [3,4,19,24,25] so that this genotype-phenotype correlation might not be general [18,19,26]. The c.804dupC mutation has been shown to cause both types of BPES [4,19,25], and the c.672_701dup causing polyalanine expansion most likely leads to BPES type II [19]. Missense mutations have been associated with both BPES type I [17] and II [3,19]. The patients from families A and B in this study, with novel c.241T>C and c.650C>G mutations, respectively, had type II BPES. The c.650C>G mutation is the first mutation described that occurs immediately upstream of the polyalanine domain and associated with type II BPES. This may raise a possibility that the region containing the c.650C>G mutation is of importance for FOXL2 function.

The c.672_701dup (p.Ala224_Ala234dup) was found in families D, E, and F (Table 1), consistent with a mutation hotspot. To check the origin of the c.672_701dup mutation in three families (families D, E, and F in Figure 3), six SNPs

| Family | Patient | Upstream of FOXL2 | Inside FOXL2 | Downstream of FOXL2 |
|--------|---------|------------------|-------------|-------------------|
|        |         | rs11924939       | rs28937885  | rs7432551         | rs28937884 | rs13325788 | rs2291252 |
| d      | II:1    | C                | T           | C                 | T         | G         | C         |
| e      | III:1   | C                | T           | C                 | T         | G         | T         |
| f      | II:1    | C                | T           | C                 | T         | G         | C         |

The origin of the c.672_701dup mutation in family D is different from family E as they have different SNP at rs2291252. This mutation in family F is a de novo event, although patient II:1 in family F shares the same haplogroup of the six SNPs with that of family D.

Figure 4. Multiple alignment of 11 FOXL2 orthologs. This demonstrated high conservation of residues involved by the p.Tyr81His and p.Ser217Cys mutation. The resource for the 11 FOXL2 orthologs was as follows: human (Homo sapiens, NP_075555), mouse (Mus musculus, NP_036150), pig (Sus scrofa, AAQ91845), rabbit (Oryctolagus cuniculus, AAQ91846), rat (Rattus norvegicus, XP_345976), mole vole (Ellobius lutescens, AAV30684), cow (Bos taurus, NP_001026920), goat (Capra hircus, AAM52099), chicken (Gallus gallus, NP_001012630), zebrafish (Danio rerio, XP_698915), and salmon trout (Oncorhynchus mykiss, AAS7040).
The human FOXL2 mutation database. Hum Mutat 2004; 24:189-93.
9. Or SF, Tong MF, Lo FM, Lam TS. Three novel FOXL2 gene mutations in Chinese patients with blepharophimosis-ptosis-epicanthus inversus syndrome. Clin Med J (Engl) 2006; 119:49-52.
10. Tang S, Wang X, Lin L, Sun Y, Wang Y, Yu H. Mutation analysis of the FOXL2 gene in Chinese patients with blepharophimosis-ptosis-epicanthus inversus syndrome. Mutagenesis 2006; 21:35-9.
11. Qian X, Shu A, Qin W, Xing Q, Gao J, Yang J, Feng G, He L. A novel insertion mutation in the FOXL2 gene is detected in a big Chinese family with blepharophimosis-ptosis-epicanthus inversus. Mutat Res 2004; 554:19-22.
12. Smith DW. Recognizable patterns of human malformation: genetic, embryologic, and clinical aspects. Major Probl Clin Pediatr 1970; 7:1-368.
13. Smith RJ, Holcombe JD, Daiger SP, Caskey CT, Pelias MZ, Alford BR, Fontenot DD, Hejtmancik JF. Exclusion of Usher syndrome gene from much of chromosome 4. Cytogenet Cell Genet 1989; 50:102-6.
14. Zhang Q, Minoda K. Detection of congenital color vision defects using heteroduplex-SSCP analysis. Jpn J Ophthalmol 1996; 40:79-85.
15. Small KW, Stalvey M, Fisher L, Mullen L, Dickel C, Beadles K, Reimer R, Lessner A, Lewis K, Pericak-Vance MA. Blepharophimosis syndrome is linked to chromosome 3q. Hum Mol Genet 1995; 4:443-8.
16. Cocquet J, De Baere E, Gareil M, Pannetier M, Xia X, Fellous M, Veitia RA. Structure, evolution and expression of the FOXL2 transcription unit. Cytogenet Genome Res 2003; 101:206-11.
17. Dollfus H, Stoetzel C, Riehm S, Lahlou Boukoffa W, Bediard Boulaneb F, Quillet R, Abu-Eid M, Speeg-Schatz C, Francfort JJ, Flament J, Veillon F, Perrin-Schmitt F. Sporadic and familial blepharophimosis -ptosis-epicanthus inversus syndrome: FOXL2 mutation screen and MRI study of the superior levator eyelid muscle. Clin Genet 2003; 63:117-20.
18. Udar N, Yellore V, Chalukya V, Yelchits S, Silva-Garcia R, Small K. FOXL2 Consortium. Comparative analysis of the FOXL2 gene and characterization of mutations in FOXL2 patients. Hum Mutat 2003; 22:222-8.
19. De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon M, Fellous M, Fryns JP, Garza A, Jonsrud C, Koivisto KA, Krause A, Leroy BP, Meire F, Pomp A, Van Maldergem L, De Paepe A, Veitia R, Messiaen L, FOXL2 and BR, Fontenot DD, Hejtmancik JF. Exclusion of Usher syndrome gene from much of chromosome 3q. Cytogenet Cell Genet 1992; 60:47-52.
20. Kumar A, Babu M, Raghunath A, Venkatesh CP. Genetic analysis of a five generation Indian family with BPES: a novel missense mutation (p.Y215C). Mol Vis 2004; 10:445-9.
21. Cabaret S, Demarez A, Moumne L, Fellous M, De Baere E, Veitia RA. A recurrent polyalanine expansion in the transcription factor FOXL2 induces extensive nuclear and cytoplasmic protein aggregation. J Med Genet 2004; 41:932-6.
22. Moumne L, Fellous M, Veitia RA. Deletions in the polyAlanine-containing transcription factor FOXL2 lead to intranuclear aggregation. Hum Mol Genet 2005; 14:3557-64.
23. Harris SE, Chand AL, Winship IM, Gersak K, Aitomaki K, Shellong AN. Identification of novel mutations in FOXL2 associated with premature ovarian failure. Mol Hum Reprod 2002; 8:729-33.
24. Fokstuen S, Antonarakis SE, Blouin J. FOXL2-mutations in
blepharophimosis-ptosis-epicanthus inversus syndrome (BPES); challenges for genetic counseling in female patients. Am J Med Genet A 2003; 117:143-6.

25. Kosaki K, Ogata T, Kosaki R, Sato S, Matsuo N. A novel mutation in the FOXL2 gene in a patient with blepharophimosis syndrome: differential role of the polyalanine tract in the development of the ovary and the eyelid. Ophthalmic Genet 2002; 23:43-7.

26. Bell R, Murday VA, Patton MA, Jeffery S. Two families with blepharophimosis/ptosis/epicanthus inversus syndrome have mutations in the putative forkhead transcription factor FOXL2. Genet Test 2001; 5:335-8.

27. Ramirez-Castro JL, Pineda-Trujillo N, Valencia AV, Muneton CM, Botero O, Trujillo O, Vasquez G, Mora BE, Durango N, Bedoya G, Ruiz-Linares A. Mutations in FOXL2 underlying BPES (types 1 and 2) in Colombian families. Am J Med Genet 2002; 113:47-51.

28. Vincent AL, Watkins WJ, Sloan BH, Shelling AN. Blepharophimosis and bilateral Duane syndrome associated with a FOXL2 mutation. Clin Genet 2005; 68:520-3.

29. Cha SC, Jang YS, Lee JH, Kim HK, Kim SC, Kim S, Back SH, Jung WS, Kim JR. Mutational analysis of forkhead transcriptional factor 2 (FOXL2) in Korean patients with blepharophimosis-ptosis-epicanthus inversus syndrome. Clin Genet 2003; 64:485-90.