To the Editor: Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; OMIM#110100) is featured by malformation of the eyelid, including ptosis, epicanthus inversus, telecanthus, and reduction of the horizontal fissure length with a prevalence of 1 in 50,000.1-3 If not well treated, BPES could result in strabismus and amblyopia.4-6 So far, BPES has been divided into two categories: Type I is characterized by ocular symptoms with premature ovarian failure (POF), while POF is absent in Type II.5,9

Human forkhead box L2 ( FOXL2 ) (OMIM#605597) belongs to the forkhead transcription factor family.4 The FOXL2 protein is mainly expressed in fetal and adult granulosa cells in the ovary, functioning as an important regulator in embryonic development of the ovaries and eyelids.3 The FOXL2 gene consists of a single exon of 2.7 kb located at chromosome 3q23, encoding 376 amino acids, including a 100-amino acid DNA-binding FKH domain and a polyalanine tract. Moreover, FOXL2 has been recognized to be an important regulator of lipid metabolism, reactive oxygen species detoxification, and carcinogenesis.2

To date, over 115 BPES-related mutations in over 210 BPES patients have been identified.7 Intragenic mutations of the FOXL2 gene take up the biggest part (71%) of the genetic defects in BPES.8 Frameshift mutations, nonsense mutations, and missense mutations are all observed in the FOXL2 gene.9 Furthermore, around 17% of indel FOXL2 mutations are located outside its transcription unit.10,11 Typically, mutations causing truncation the protein before the polyalanine tract usually give rise to Type I, while mutations that extend the protein usually linked with Type II.10,11 Furthermore, our previous study has proved that FOXL2 mutation results in the dysfunction as repressor to regulate steroidogenic acute regulatory protein (StAR) as to contribute to the pathogenesis of BPES Type I.3

In this study, we presented, in detail, the clinical characteristics of our previous study has proved that FOXL2 mutation results in the dysfunction as repressor to regulate steroidogenic acute regulatory protein (StAR) as to contribute to the pathogenesis of BPES Type I.3

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Complementary DNA (cDNA) encoding wild-type (WT) FOXL2 was prepared by PCR using primers incorporating restriction enzyme sites. The DNA fragment amplified from the WT gene and mutant (MT) gene was cloned into digested pseudo-cDNA (pcDNA) 3.1 and EGFP-N1 plasmids, producing pcDNA3.1-FOXL2-WT/MT and N1-FOXL2-EGFP-WT/MT.

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Twenty-four hours before transfection, 293T cells were seeded into 6-cm dishes (1 × 10^5 cells/dish) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, CA, USA) containing 10% fetal calf serum (Gibco-Invitrogen, Grand Island, NY, USA) and 1% penicillin/streptomycin and maintained at 37°C in a 5% CO_2 atmosphere. The 293T cells were transfected with N1-FOXL2-EGFP, N1-FOXL2-WT-EGFP, or N1-FOXL2-MT-EGFP using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Subcellular localization/aggregation was observed after 72 h of transfection by confocal laser scanning microscopy.

To evaluate StAR and SIRT1 gene expression, quantitative SYBR Green real-time (RT) PCR was performed on an ABI 7300 system. Murine Leydig tumor cell line-1 (MLTC-1) cells in six-well plates were transfected with 4 μg of pcDNA3.1 expression vector with WT or mutant FOXL2 cDNAs and an empty pcDNA3.1 vector using Lipofectamine 2000 reagent. After 48 h, the cells were cultured in DMEM supplemented with 2% bovine serum albumin for 2 h. Total mRNA was then extracted from the cells using TRIzol (Invitrogen, CA, USA) according to the manufacturer’s instructions. cDNA was then synthesized in a 20 μl mixture. RT-PCR was performed with 2 μg RNA using SuperScript II (Invitrogen, CA, USA). The housekeeping gene GAPDH was used as an endogenous control.

Patients with BPES Type II presented with two alleles: a pathogenic allele and a WT allele [Figure 1c]. Sequencing of the coding sequence of the FOXL2 gene uncovered a novel compound mutation (c.112_151 del, c.158_159 insCGCG) [Figure 1d]. This mutation was not present in 100 normal subjects or in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). The mutated proteins, FOXL2-MT, replaced 16 amino acids (38th to 53th Aa) with RRTR, retaining functional FKH domain and polyalanine tract [Figure 2a and 2b]. The detailed protein sequence was listed in Supplementary Figure 1. Typically, shortened FOXL2 leads to Type I BPES. Thus, it is important to determine the mutated FOXL2 activity as a transcript factor.

To further investigate the effect of the mutation on the subcellular localization of the FOXL2 protein, we conducted localization studies.

### Table 1: Clinical features of the Chinese families with BPES

| Patient | Age (years) | IICD (mm) | IPFH (mm) | HPFL (mm) | Levator function (mm) |
|---------|-------------|-----------|-----------|-----------|-----------------------|
| I-4     | 63          | 33        | 4         | 24        | 2                     |
| II-2    | 38          | 31        | 3         | 25        | 2                     |
| III-1   | 9           | 22        | 3         | 25        | 2                     |

HPFL: Horizontal palpebral fissure length; IICD: Inner intercanthal distance; IPFH: Vertical interpalpebral fissure height; LE: Left eye; RE: Right eye; BPES: Blepharophimosis-ptosis-epicanthus inversus syndrome.

*Figure 1:* (a) Three-generation BPES Type I pedigree. (b) Photographs of the ocular region of the BPES families. (c) The PCR products amplified by PCR from samples from BPES patient and unaffected family member. Gel electrophoresis of the PCR products from the BPES patients revealed two fragments. The unaffected individuals contained a single fragment. The left and right lane is the DNA marker (100 bp). (d) Genomic analysis of the cloned PCR products of the FOXL2 gene, which was inserted into the multiple cloning site (EcoRI) of the pGEM-T easy vector. This analysis reveals the FOXL2 mutation found in affected members of this BPES Type I family. This variant was absenting in 100 control individuals, including 3 relatives of the affected families. BPES: Blepharophimosis- ptosis-epicanthus inversus syndrome; PCR: Polymerase chain reaction.
in 293T cells. The cells were transfected with N1-FOXL2-WT-EGFP or N1-FOXL2-MT-EGFP. Through the observation of recombinant protein, we found that both mutant and wild-type FOXL2 are distributed in the nucleus [Figure 3a]. The result indicated that the mutation of FOXL2 may retain its function as a transcription factor.

To further verify that FOXL2 regulated gene, StAR and SIRT1 expression, we performed RT-PCR analyses to measure the endogenous mRNA expression of the StAR and SIRT1 gene following stimulation with both the WT and mutant FOXL2 proteins. The MLTC-1 cells transfected with the mutated FOXL2 showed the same endogenous StAR and SIRT1 expression as wild-type group. However, the expression of mutant FOXL2 or wild-type FOXL2 was significantly downregulated than empty vector group [Figure 3b and 3c].

FOXL2 is an evolutionarily conserved transcription factor, identified as a key regulator of sex determination, reproductive system maturation, and eyelid development. Moreover, it has also been shown that FOXL2 also plays a key role in the pathogenesis of polycystic ovary syndrome, keloid, and tumorigenesis. It is to note that one allele mutation in FOXL2 could result in decreased expression and the mutation of both FOXL2 alleles could be lethal. Therefore, typical BPES patients contain the heterozygous mutation.

To date, over 110 mutations have been identified in 210 families with BPES worldwide. Before any mechanism studies were conducted, Type I BPES was hypothesized to result from genomic truncation of FOXL2 gene. Polyalanine expansion represents a common type of in-frame mutation; these types of mutations account for 30% of the reported mutations in the FOXL2 ORF and often give rise to Type II BPES. Currently, the new classification scheme for BPES is based on whether the FOXL2 mutation will disrupt the protein’s function as a transcription factor, causing the loss of its regulatory control over target genes related to ovarian development, such as SIRT1 or StAR.

The major problem for Type I BPES female patients, however, is infertility rather than eyelid malformation. For Type II patients, the major treatment is performed to solve ocular complications as to avoid the occurrence of strabismus or amblyopia. Thus, to identify the certain type of BPES is of great significance as to provide clinical suggestions. Here, we not only discovered that novel pattern of deletion-insertion compound FOXL2 mutation in BPES Type II patients, but also proved the novel mutation did not result in dysfunction of FOXL2 as a transcriptional repressor.

It is to note that POF is a multifactor-involved disease. Although we have tested typical FOXL2 (SIRT1 and StAR) regulating gene expression, we cannot eliminate other factors that are also regulated by FOXL2. More importantly, FOXL2 could regulate both female reproductive system and eyelid maturation. FOXL2 mutations in Type I BPES influence both ovarian and eyelid development; however, in Type II, it only leads to the dysfunction in eyelid development but preserves the ability to regulate female reproductive system development.
In conclusion, we discovered a novel heterozygous deletion-insertion mutation in Chinese families with BPES Type II. Furthermore, this is the only and first report that a deletion-insertion compound mutation in \textit{FOXL2} gene results in BPES Type II. Our work provides additional support for previously reported genotype-phenotype correlations and expands the spectrum of known \textit{FOXL2} gene mutations in BPES Type II.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

**Declaration of patient consent**

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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**Conflicts of interest**

There are no conflicts of interest.

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Supplementary Figure 1: The full-length sequence of FOXL2 in unaffected and BPES patients. BPES: Blepharophimosis-ptosis-epicanthus inversus syndrome.

| Amino acid sequence | FOXL2-WT | FOXL2-MT |
|---------------------|----------|----------|
| MNASYPEPDDAGALLPETGRTVKEP | MNASYPEPDDAGALLPETGRTVKEP | EOGPPPSFQGCGKDCQQAGTAPPPVPQPGPGKPGK |
| EGPYYVYAAMIREBAEKRLSGLQYQ | LAMARIEBAEKRLSGLQYQIAMKPPPYE | LLAMARIEBAEKRLSGLQYQIAMKPPPYE |
| YSKKPPPYVEVKOSQGNSRNLSLNEC | KNKOSQGNSRNLSLNECPIKVPREQG | KKKOSQGNSRNLSLNECPIKVPREQG |
| FKVPPREGGGGERKGNVYXTLDPECDEMFGERKGNVYXTLDPECD | GERKGNVYXTLDPECDEMFGERKGNVYXTLDPECD |
| EKQNYRFRGRMRQKFPFRPAFHCPAGK | RMKQYRFRGRMRQKFPFRPAFHCPAGK | GGCBQAGDQDQYDLAPPKYLQSGFL |
| GLPSAAGAAGDCVAGGGAGOYSYLA | NSWVPQCPSPSVYASCMAAAAAA | NNSWVPQCPSPSVYASCMAAAAAA |
| PPXQLQGFLNSWLPQPSPSQPMYPAS | NSWLPQCPSPSVYASCMAAAAAA | NSWLPQCPSPSVYASCMAAAAAA |
| CQMAAAAAAMAAGRPSPQGAAV | AAAAAAGRPSPQGAAV | AAAAAAGRPSPQGAAV |
| VKLQAPMASYQPYTRQSMALPPGVP | YGPTTRQSMALPPGVP | YGPTTRQSMALPPGVP |
| NSWQLGQPAPRPPPHPPPHPRHPRH | LNHAAAAAPPRPPPHPPPHPRHPRH | LNHAAAAAPPRPPPHPPPHPRHPRH |
| SPATAAPPAPAPTSAAPLGFAQACARGPEL | APTSAAPLGFAQACARGPELAMHCSYW | HOSATGALHIRNL |
| AMNHCKDVFQDHDKGTGALHRIKDL | | HOSATGALHRIKDL |

The full-length sequence of FOXL2 in unaffected and BPES patients. BPES: Blepharophimosis-ptosis-epicanthus inversus syndrome.