Genomic Disruption of *FOXL2* in Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome Type 2: A Novel Deletion-Insertion Compound Mutation

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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] 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]

Human forkhead box L2 (*FOXL2*) (OMIM#605597) belongs to the forkhead transcription factor family.[6] 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.[5] 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.[1] 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.[1] In this study, we presented, in detail, the clinical characteristics of a Chinese family with BPES Type II and investigated the germline *FOXL2* mutation spectrum in the affected patients.

A Chinese family with BPES Type II was ascertained through the Shanghai Xinhua Hospital of Shanghai Jiao Tong University School of Medicine. Five individuals (I-3, I-4, II-1, II-2, and III-1), including three affected individuals (I-4, II-2, and III-1), were recruited [Figure 1a]. An ophthalmologist performed detailed examinations of the patients and diagnosed BPES based on the following criteria: blepharophimosis, ptosis, epicanthus inversus, and telecanthus [Figure 1b]. We examined DNA samples from all these patients. In addition, the clinical data of the patients were examined in detail [Table 1]. The proband of the family (III:1), a 9-year-old boy, acquired the pathogenic gene from his mother. All affected patients presented with typical features of BPES Type II, including small palpebral fissure, ptosis of the eyelids, epicanthus inversus, and telecanthus, and without POF occurrence.

DNA extraction was performed as previously described. The patients' genomic DNA was extracted from peripheral blood leukocytes (51206; QIAGEN, Hilden, Germany). The region of the *FOXL2* gene was divided into three segments, and genomic fragments encompassing the *FOXL2* coding sequence were amplified using the following primers: *FOXL2-1F*: 5#-TTGAGACTTGGCCGTAAGCG-3#; *FOXL2-1R*: 5#-CTCGTTGAGGCTGAGGTTGT-3# *FOXL2-2F*: 5#-ACAACCTCAGCCTCAACGAG-3#; *FOXL2-2R*: 5#-CCAGGCCATTGTACGAGTTC-3#; *FOXL2-3F*: 5#-GCTTCCTCAACAACTCGTGGC-3#; and *FOXL2-3R*: 5#-CTGCATCCTCGCATCCGTCT-3#. Polymerase chain reaction (PCR) was performed as previously described. The PCR products were sequenced and analyzed.

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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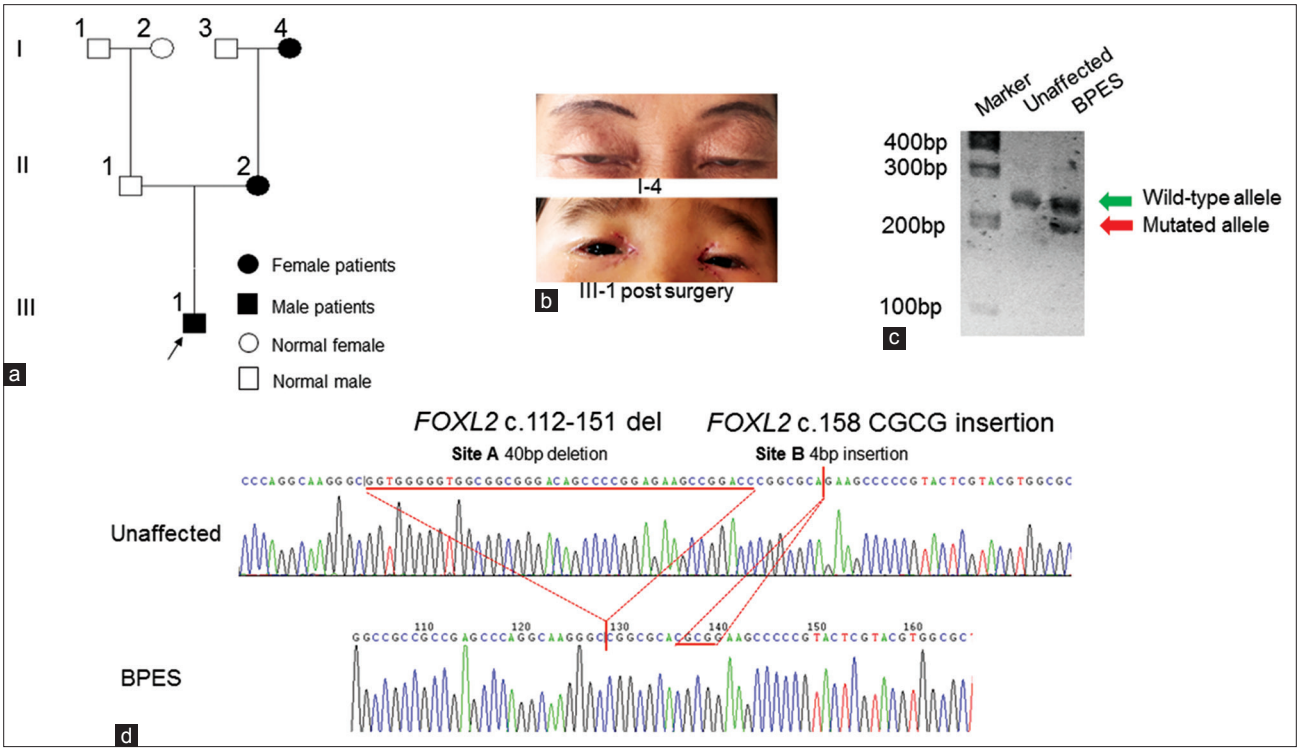

Figure 1: (a) Three-generation BPES Type I pedigree. (b) Photographs of the ocular region of the BPES families. (c) The PCR products amplified by PCR from samples from BPES patient and unaffected family member. Gel electrophoresis of the PCR products from the BPES patients revealed two fragments. The 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.

Table 1: Clinical features of the Chinese families with BPES

Patient	Age (years)	IICD (mm)	IPFH (mm)		HPFL (mm)		Levator function (mm)	
			RE	LE	RE	LE	RE	LE
I-4	63	33	4	4	24	25	2	2
II-2	38	31	4	3	25	25	2	2
III-1	9	22	2	3	25	23	2	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.

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 (38^{th} to 53^{th} 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

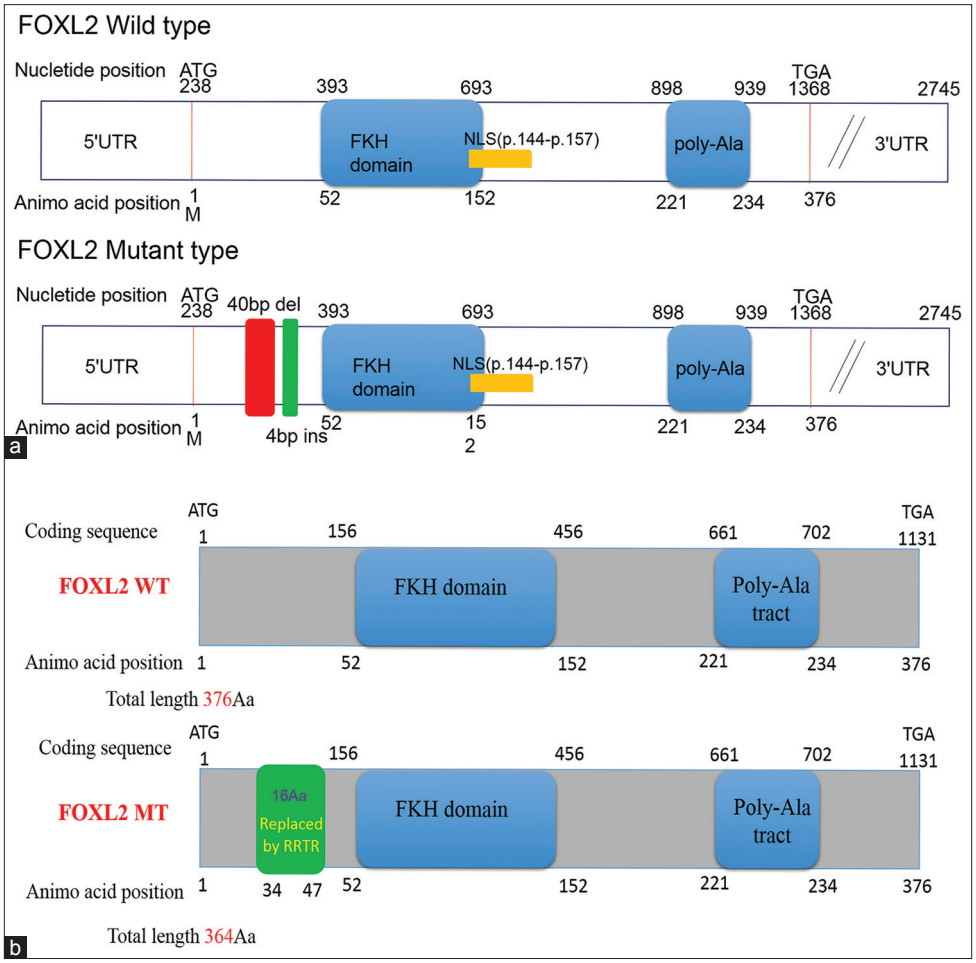

Figure 2: Computational prediction of the c.19_95 del of *FOXL2* resulting in two truncated proteins. (a) The deleted region of *FOXL2* in the BPES patients is labeled in red. (b) Computational prediction of the mutated proteins, FOXL2-MT and FOXL2-WT. BPES: Blepharophimosis-ptosis-epicanthus inversus syndrome.

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.[12] 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.[13] Therefore, typical BPES patients contain the heterozygous mutation.

To date, over 110 mutations have been identified in 210 families with BPES worldwide.[1] Before any mechanism studies were conducted, Type I BPES was hypothesized to result from genomic truncation of *FOXL2* gene.[12] 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.[1] 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 *SIRT-1* or *StAR*.[11]

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.[3] 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.[4] Although we have tested typical *FOXL2* (*SIRT1* and *StAR*) regulating gene expression, we cannot eliminate other factors that are also regulated by *FOXL 2*. 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.[12]

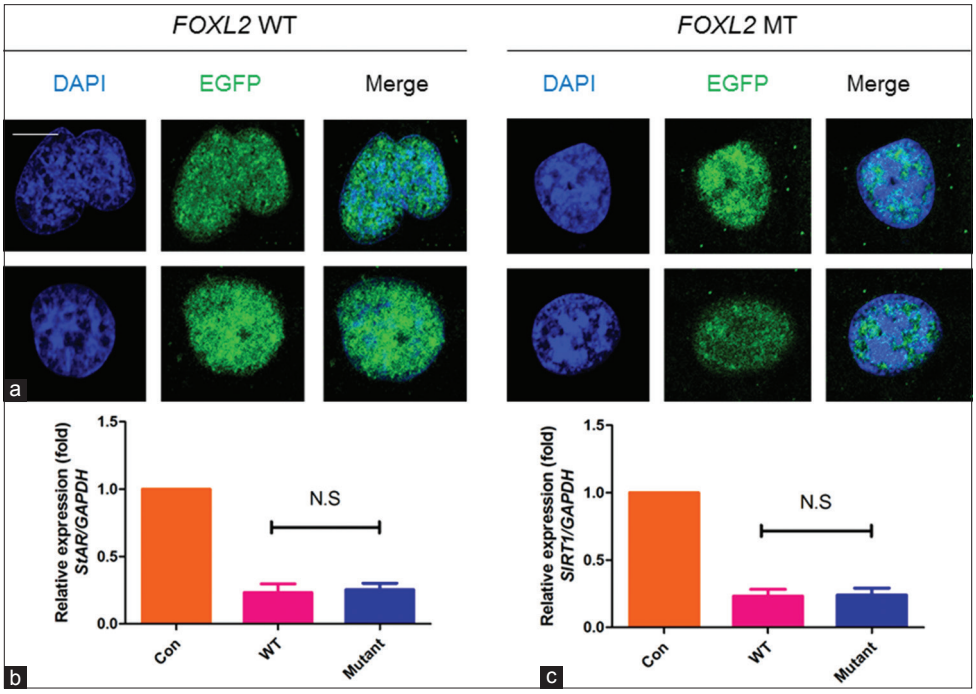

Figure 3: (a) Subcellular localization of wild-type (WT) and indel mutant FOXL2 proteins. The middle panel corresponds to the most representative subcellular localization of FOXL2 as a fusion protein with green fluorescent protein (EGFP-tagged). The left panel corresponds to Hoechst nuclear staining. The right panel is a merged image of the previous two images. Scale bar: 5 μM. (b and c) Relative *StAR* (b) and *SIRT1* (c) mRNA expression when cells were transiently transfected with the pcDNA3.1 vector, WT FOXL2 and mutant FOXL2. StAR: Steroidogenic acute regulatory protein.

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 *FOXL2* gene results in BPES Type II. Our work provides additional support for previously reported genotype-phenotype correlations and expands the spectrum of known *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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Nil.

Conflicts of interest

There are no conflicts of interest.

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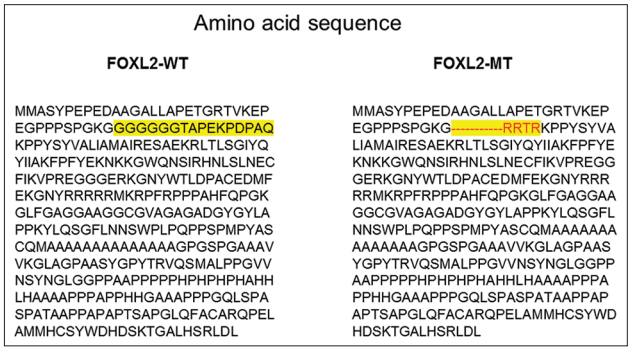

Supplementary Figure 1: The full-length sequence of *FOXL2* in unaffected and BPES patients. BPES: Blepharophimosis-ptosis-epicanthus inversus syndrome.