Mutation analysis of the FOXL2 gene in Chinese patients with blepharophimosis–ptosis–epicanthus inversus syndrome

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Blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) is an autosomal dominant disorder characterized by blepharophimosis, ptosis and epicanthus inversus. Based on the presence and absence of premature ovarian failure, two clinical types have been distinguished. Both types of BPES have been mapped to chromosome 3q23 and are mostly due to mutations of a forkhead transcription factor FOXL2 gene which locates at this region. We screened for FOXL2 mutations in Chinese patients with BPES. A novel mutation (g.901–930dup30) which could result in an expansion of the polyalanine tract was found in two BPES type II families and one sporadic case. In addition, a new g.952delC mutation was identified in two patients from a BPES family of undetermined type. The previously reported g.892C>T (p.Q219X) was also found in 12 patients from a large BPES family of type I. No mutations were detected in three other BPES families and three sporadic cases. So we speculate that in a fraction of the BPES patients the genetic defect may represent a change in gene dosage or a rearrangement outside the transcription unit of FOXL2.

Introduction

Blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) (MIM110100) is a rare autosomal dominant disorder mainly characterized by blepharophimosis (shortened palpebral fissures), ptosis (drooping eyelids) and epicanthus inversus (a vertical skin fold arising from the lower eyelid). Two clinical types have been distinguished: type I, characterized by eyelid defects with infertility in affected females due to premature ovarian failure (POF) and type II, characterized by eyelid anomalies only (1).

According to cytogenetic rearrangements, linkage analysis and positional cloning, BPES were mapped to chromosome 3q23 (2,3). BPES was shown to be caused by mutations in the forkhead transcription factor gene FOXL2, which locates at this region (4). FOXL2 is a single-exon gene of 2.7 kb, predicting a protein of 376 amino acids. Containing a 100-amino acid DNA-binding forkhead domain, FOXL2 belongs to the large family of forkhead transcription factors whose mutations are known to be responsible for a number of hereditary disorders. The expression of FOXL2 in mesenchyme of the developing eyelids and in ovarian follicular cells suggests FOXL2 might be a key regulator in early development of the eyelids and ovary and in maintenance of the vertebrate female gonad (4,5).

In our present study, we performed mutation analysis of FOXL2 in Chinese patients with BPES. We used polymerase chain reaction to amplify the open reading frame (ORF) and 5′untranslated region (5′UTR) of FOXL2, followed by sequencing.

Materials and methods

Patients

The 26 subjects involved 22 patients from 3 BPES families of type II, 1 large BPES family of type I, 2 families with BPES whose BPES type remained undetermined, 4 sporadic cases and 100 healthy individuals including 35 relatives of the affected families [Figures 1 and 2 (6)]. The BPES patients were diagnosed as follows: blepharophimosis, ptosis and epicanthus inversus; POF (in BPES type I) was defined as cessation of menses for a duration of >6 months at age <40 years and an FSH concentration of >40 IU/L. Patients with POF underwent a clinical assisted examination that included taking menstrual history, pelvic B-ultrasonic and hormone levels. Additional information such as other malformations and defects were also ascertained whenever needed. Written consents were obtained from all the patients, their relatives and other healthy control people.

Genomic DNA extraction

Genomic DNA extraction kits (Shanghai Sangon Biological Engineering & Technology Services Co., Ltd) (SSBE Sangon) were used to extract genomic DNA from peripheral blood leukocytes according to the manufacturer’s instructions.

PCR amplification/PCR product cloning

The PCR amplification kits and PCR product cloning kits were provided by SSBE Sangon. The PCR primers were designed as described by Crisponi et al. and De Baere et al. (4,7) (as shown in Table I).

The PCR reactions were performed in a 25 μl volume containing 100 ng genomic DNA, 10% volume dimethylsulphoxide (DMSO), 0.5 μM primers, 1.5 mM MgCl2, 200 μM dNTP, 1U Taq, and 2.5 μl 10 × PCR buffer. Cycling was carried out with an initial denaturation step of 95°C for 3 min, followed by 6 cycles of 92°C for 1 min, 60°C for 1 min and 72°C for 1 min; then 30 cycles of 92°C for 45 s, 60°C for 45 s and 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were separated by 1% agarose gel electrophoresis, then purified with Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced directly. Because the products amplified by primers E and F could not be sequenced correctly, we purified those fragments and cloned into pUCm-T vector (SSBE Sangon). Then the constructs were used to transform the competence DH5α Escherichia coli cells. The plasmids were extracted from the positive colonies and sequenced subsequently.

Sequencing

All the products were sequenced by SSBE Sangon with ABI PRISM 377-96 sequencer. The sequencing primers of PCR products were the same as PCR amplification, whereas the primers of the recombinant plamids which contained the target fragments were M13.

Results

Sequence electropherograms of FOXL2

In this study, mutation analysis was carried out in 26 patients both from BPES families and sporadic cases. The sequence results were compared with the putative FOXL2 ORF and 5′UTR by Genbank Blastn software. All the fragments which
Fig. 1. Pedigree for BPES families and sporadic patients: affected individuals are indicated by filled symbols, individuals tested were labelled by inverted triangle, the probands were marked with upwards arrow, the mutation of F2G was reported by Qian et al. (6). Abbreviations: F1 = BPES type I, F2 = BPES type II, F = BPES type undetermined, C = sporadic cases.
contained the mutations were confirmed by the cloning and sequencing of at least six clones. All changes described are heterozygous changes and were not detected in 100 control individuals.

A previously reported g.892C>T (8) mutation was found in the 12 affected members of a large BPES family of type I, and this mutation results in a truncation of the putative protein downstream of the forkhead domain (as shown in Figure 3a and b).

A novel 30 bp duplication (g.901–930dup30) was found in four patients of families B and C and a sporadic case in C. This mutation causes the expansions of 10 alanine residues (p.222–231dup10) in the polyalanine (polyAla) tract. However, this mutation was absent in unaffected individuals within the three families (as shown in Figure 3c and d).

A new g.952delC mutation was found in patients of FF, which leads to a frameshift at codon 239, resulting in a truncated protein at codon 270 (as shown in Figure 3e and f).

No mutations were found in other BPES families and three sporadic cases by this way.

**Predicted proteins of FOXL2 mutations**

According to the effect on the predicted protein (NCBI ORF finder), De Baere et al. have subdivided the FOXL2 mutations in classes (7). Groups A–D contain the predicted truncated proteins: without forkhead domain (group A), with partial forkhead domain (group B), with complete forkhead domain and without polyAla tract (group C), and with complete forkhead and polyAla domains (group D). Group E comprises frameshift mutations that lead to elongated proteins with complete forkhead and polyAla domains. Group F contains in-frame changes, and group G contains missense mutations.

Group H comprises the cytogenetic rearrangements. The g.901–930dup30 mutation belongs to group F because this mutation leads to elongated polyAla tract. The g.952delC mutation leading to a truncated protein with complete forkhead and polyAla domains belongs to group D. The g.892C>T mutation belongs to group C, leading to a truncated protein without polyAla tract (as shown in Figure 4).

**Discussion**

Many mutation sites which reside within the ORF of the FOXL2 have been reported, as described in the human FOXL2 mutation database by Beysen et al. (9). The reported total number of distinct mutations in the FOXL2 gene is 53 so far. To our knowledge, the g.901–930dup30 mutation detected in two BPES families of type II and a sporadic case and the g.952delC mutation in one BPES family of undetermined type have not been reported previously. Both of them are novel mutations.

The g.901–930dup30 mutation detected in the two BPES families and a sporadic case is an in-frame mutation which leads to a polyalanine expansion and then to an extended protein (p.222–231dup10). The number of alanine residues is strictly conserved through mammalian evolution, and expansions from 14 to 24 residues account for 30% of the reported mutations and lead mainly to BPES type II (7). The mechanism for the molecular pathogenesis of the polyAla expansions of FOXL2 may be its mislocalization concomitant with its inclusion into nuclear aggregates which may diminish the pool of active protein (10). Several transcription factor genes form α-helices in the alanine-rich regions which were found to be responsible for the repression of target genes (11). Up to now, polyAla expansions have been known to cause at least nine human diseases, including mental retardation and malformations of the brain, digits and other structures (12,13).

The g.952delC mutation leads to a frameshift, resulting in the predicted protein 107 amino acid residues shorter than the wild-type protein. The amino acids of the altered C-terminus are not homologous to any sequences in the GenBank, as determined by a BLAST search. Because both of the two patients in this family are male, the type could not be assessed. The g.952delC mutation may cause a frame shift at codon 239 that is downstream of the forkhead domain, leading to truncation of the coding region at codon 270. It is likely that the deletion in this family may be involved in the pathogenesis of BPES. Truncations of FOXL2 downstream of the forkhead domain may act in a dominant-negative fashion by producing proteins that bind to DNA but fail to activate transcription.
Fig. 3. Sequence electropherogram of FOXL2. (a) The normal sequence result corresponding to the region of h. (b) The g.892C>T mutation in all affected family members of F1A. (c) The normal sequence result corresponding to the region of d. (d) The novel g.901–930dup30 mutation in patients of F2A and F2B and CC. (e) The normal sequence result corresponding to the region of f. (f) The g.952delC mutation in patients of FF.
The nonsense mutation g.892C>T reported previously by De Baere et al. (8) was also identified by us in the large BPES type I family. This mutation results in the production of a truncated protein missing the normal carboxy-terminal sequences and causing FOXL2 haploinsufficiency. Furthermore, this protein may exert a dominant-negative effect by disabling the transcriptional repressor activity of wild-type FOXL2, so as to increase the expression of steroidogenic acute regulatory (StAR) gene (14) and other follicle differentiation genes in small and medium follicles to accelerate follicle development, resulting in increased initial recruitment of dormant follicles and thus the POF phenotype.

In a previous mutation study (7), it was concluded that 67% of the BPES patients have intragenic FOXL2 mutations. According to the mutations, a correlation between the genotype and the phenotype of BPES has been assumed: for proteins with a truncation before the polyalanine tract, the risk for development of POF is high; mutations that lead to a predicted truncated or extended protein containing an intact forkhead domain induces extensive nuclear and cytoplasmic protein aggregation. The normal and mutated predicted protein translations of FOXL2 are shown in Fig. 4.

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References


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