



Genetic analysis of a five generation Indian family with BPES: A novel missense mutation (p.Y215C)

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Purpose: Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) is a rare eye genetic disorder caused by mutations in the *FOXL2* gene located at chromosome 3q23. The purpose of the present study was to carry out genetic analysis of BPES in a five-generation Indian family.

Methods: Peripheral blood samples were obtained from individuals for genomic DNA isolation. To determine the linkage of this family to the *FOXL2* locus, haplotype analysis was carried out using microsatellite markers from the BPES candidate region. Five overlapping sets of primers were used to amplify the entire coding region of the *FOXL2* gene for mutation detection. Allele-specific oligonucleotide hybridization (ASOH) analysis was carried out to determine segregation of the mutation in the family and to also determine if the mutation was present in 100 ethnically matched normal control chromosomes.

Results: Pedigree analysis suggested that BPES segregated in this family as an autosomal dominant trait. Cytogenetic analysis in one patient did not reveal any rearrangement. Haplotype analysis suggested that this family was linked to the *FOXL2* locus on chromosome 3q23. DNA sequence analysis showed that the BPES phenotype in this family was caused by a novel missense mutation, c.881A->G (p.Y215C).

Conclusions: This study reports for the first time a novel missense mutation in a five-generation Indian family with BPES. A review of the literature showed that the total number of mutations in the *FOXL2* gene described to date is 42.

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; OMIM 110100) is a rare eye genetic disorder that results in dysplasia of the eyelids. Patients with BPES have a combination of congenital anomalies of the eyelids characterized by reduction of the horizontal fissure length, congenital ptosis, and epicanthus inversus. BPES patients have a variable degree of epicanthus, a broad nasal bridge, and telecanthus is the most consistent finding [1]. BPES either occurs sporadically or segregates as an autosomal dominant trait in families. The frequency of BPES is estimated to be 1 in 50,000 [2]. Based on the presence and absence of premature ovarian failure (POF), BPES has been classified into type I with infertility in affected females and type II with fertility in females and males [3]. Linkage analysis and chromosome aberrations in affected patients have indicated the location of a BPES locus on chromosome 3q23 region [4-8]. Crisponi et al. [9] have recently cloned a forkhead transcription factor gene, *FOXL2* from the region and found mutations in this gene in five familial and two sporadic patients with BPES. The 2.7-kb cDNA of the *FOXL2* gene corresponds to a single exon and contains an ORF which codes for a protein of 376 amino acids [9]. *FOXL2* protein contains a characteristic monomeric 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,

which is believed to be responsible for transcriptional repression activity [10]. Northern blot analysis on multiple tissue blots has shown a single 2.7 kb band exclusively in adult ovarian mRNA [9]. In mice, the *FOXL2* gene is selectively expressed in the mesenchyme of developing mouse eyelids and adult ovarian follicles, but not in the oocyte of the adult mouse ovary, consistent with the presumed role of *FOXL2* protein in early eyelid development and ovarian maintenance [9]. Genetic analysis of BPES patients with different ethnic backgrounds has revealed several mutations in the *FOXL2* gene. However, there is no report of genetic analysis of BPES in Indian patients. Here we report for the first time genetic analysis of BPES in a five-generation Indian family.

METHODS

Family: We have ascertained a five-generation Muslim family IIS-100 (Figure 1A) with BPES from the state of Karnataka, south India. A total of eight individuals including five affected individuals were recruited for the study. All family members were examined in detail by clinicians and the patients with BPES were diagnosed according to previously established criteria [11]. Informed consent was obtained for research following the guidelines of the Indian Council of Medical Research, New Delhi.

Genetic analysis: In order to determine if BPES in this family is caused by a chromosomal rearrangement, peripheral blood sample from individual II-1 (Figure 1A) was collected in a heparinized Vacutainer tube™ (Beckton-Dickinson, Franklin Lakes, NJ) and used for high-resolution GTG-band-

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ing according to method of Yunis [12]. Peripheral blood samples were also collected in EDTA Vacutainer™ tubes (Beckton-Dickinson) for genomic DNA isolation. Genomic DNA samples were isolated from peripheral blood samples using a Wizard® Genomic DNA extraction kit (Promega Inc., Madison, WI). In order to see if this family had a mutation in the *FOXL2* gene located at the BPES locus at 3q23, six microsatellite markers (D3S1278-10 cM-D3S1267-11.2 cM-RHO-1.9 cM-ACPP-3.1 cM-D3S1238-7.7 cM-D3S1309) were selected from the BPES candidate region as reported by Small et al. [5] and used to genotype the family. The genotyping was carried out as described in Venkatesh et al. [13]. For mutation analysis, the entire coding region of the *FOXL2* gene was sequenced using five overlapping sets of primers. Primer sequences for three of the five sets (C-D, E-F, G-H) have been given in Crisponi et al. [9]. We have replaced the primer set A-B of Crisponi et al. [9] with two primer sets, FOXL1F-FOXL1R and FOXL2F-FOXL2R, as primer set A-B did not work in our hands. The primer sequences for these sets are as follows: FOXL1F: 5'-aag cgg act cgt gcg ccc caa-3'; FOXL1R: 5'-GGT AGA TGC CGG ACA GCG TGA G-3'; FOXL2F: 5'-GTG GCG CTC ATC GCC ATG GCG A-3'; and, FOXL2R:

5'-CGG CCG GAA GGG CCT CTT CAT GC-3'. Primer sequences in lower case and upper case letters are from intronic and exonic sequences, respectively. Annealing temperatures and PCR conditions for the primer sets C-D, E-F, and G-H are given in Crisponi et al. [9]. The annealing temperatures for primer sets, FOXL1F-FOXL1R and FOXL2F-FOXL2R are 67 °C and 62 °C, respectively. Both of these primer sets work in the presence of 10% DMSO. Since the *FOXL2* gene is highly GC-rich, most of the ordinary Taq DNA polymerases from several vendors in our hands failed to amplify this gene (data not shown). In our experience, we have found that the BD Advantage 2 Polymerase Mix (catalog number 8431-1) from BD Biosciences Clontech (Palo Alto, CA) was a good polymerase mix to amplify this gene. For mutation detection, PCR amplified products were purified using a Wizard® PCR Preps DNA Purification System (Promega Inc.,) and subjected to direct sequencing on an ABIprism A370 automated sequencer. Allele-specific oligonucleotide hybridization (ASOH) analysis was used to determine if the mutation segregated in the family and was not present in 100 ethnically matched normal control chromosomes as described in Cormand et al. [14].

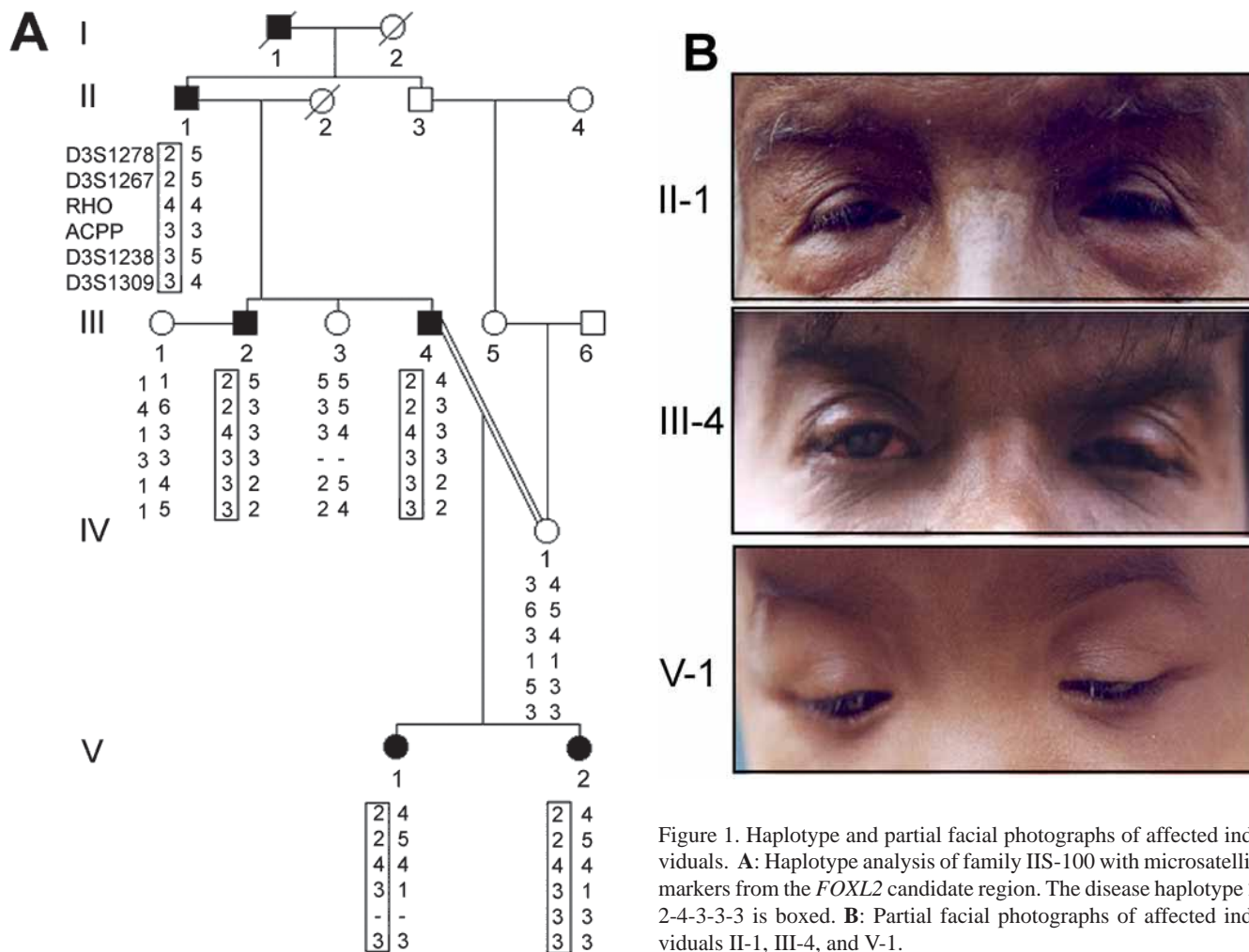


Figure 1. Haplotype and partial facial photographs of affected individuals. **A**: Haplotype analysis of family IIS-100 with microsatellite markers from the *FOXL2* candidate region. The disease haplotype 2-2-4-3-3-3 is boxed. **B**: Partial facial photographs of affected individuals II-1, III-4, and V-1.

RESULTS & DISCUSSION

All affected individuals from the family IIS-100 exhibited typical BPES features of small palpebral fissures, ptosis of the eyelids, and epicanthus inversus. All the family members had normal visual acuity and normal ocular (anterior and posterior segment) examination including normal mobility. All the patients except individual III-2 had chin elevation and telecanthus was present in all the patients. Levator function was decreased in all the patients. None of the patients exhibited microcephaly or mental retardation. Systemic examination was otherwise normal in all the patients. Figure 1B shows partial facial photographs of individual II-1, III-4, and V-1.

Visual inspection of the pedigree suggested that BPES is segregating as an autosomal dominant trait in the family IIS-100 (Figure 1A). Chromosome G-banding analysis from individual III-4 did not show any chromosomal rearrangement (data not shown), suggesting that the BPES in this family is caused by a mutation. Haplotype analysis using six microsatellite markers selected from the candidate region of the BPES locus showed that the disease haplotype 2-2-4-3-3-3 at marker loci D3S1278, D3S1267, RHO, ACPP, D3S1238, and D3S1309 co-segregated with the disease in this family, suggesting that BPES in this family is caused by a mutation in the *FOXL2* gene. In order to determine the exact nature of the mutation, the entire coding region of the *FOXL2* gene from individual III-2 was amplified in five overlapping PCR fragments and sequenced. Direct sequence analysis of PCR fragments by primer sets C-D and G-H from individual III-2 re-

vealed an A->G change at nucleotide position 881 (c.881A->G, the nomenclature used conforms to the Human Genome Variation Society suggested usage) changing codon position 215 from tyrosine to cysteine (Figure 2A,B). ASOH analysis showed that this change segregated with the disease in this family (Figure 2C). This change was not present in 100 ethnically matched control chromosomes (data not shown), suggesting that the c.881A->G change represents a mutation. In addition, the pathogenic nature of this mutation also comes from the fact that tyrosine is conserved at amino acid position 215 in different *FOXL2* orthologs (Figure 2D).

A total of 41 mutations have been reported so far in the *FOXL2* gene in patients from different countries [1,9,10,15-21]. The mutation, c.881A->G (p.Y215C) reported here is a novel missense mutation. The total number of mutations now is 42 with inclusion of the novel mutation reported in this study. Of 42 mutations, 11 are deletions, seven are duplications, six are insertions, one is a triplication, 11 are nonsense, and six are missense mutations [1,9,10,15-21]. In addition to these mutations, five chromosomal rearrangements such as microdeletions and reciprocal translocations disrupting the *FOXL2* gene function have also been reported [7-9,17,22]. Of six missense mutations reported, four (I84S, H104R, L106F, and N109K) are located in the forkhead domain [1,17,19], whereas the missense mutation (S217F) reported by De Baere et al. [10] in a Belgian patient and Y215C reported in this study are located in the region flanked by the forkhead and alanine-rich domains. Since the role of this region is not known,

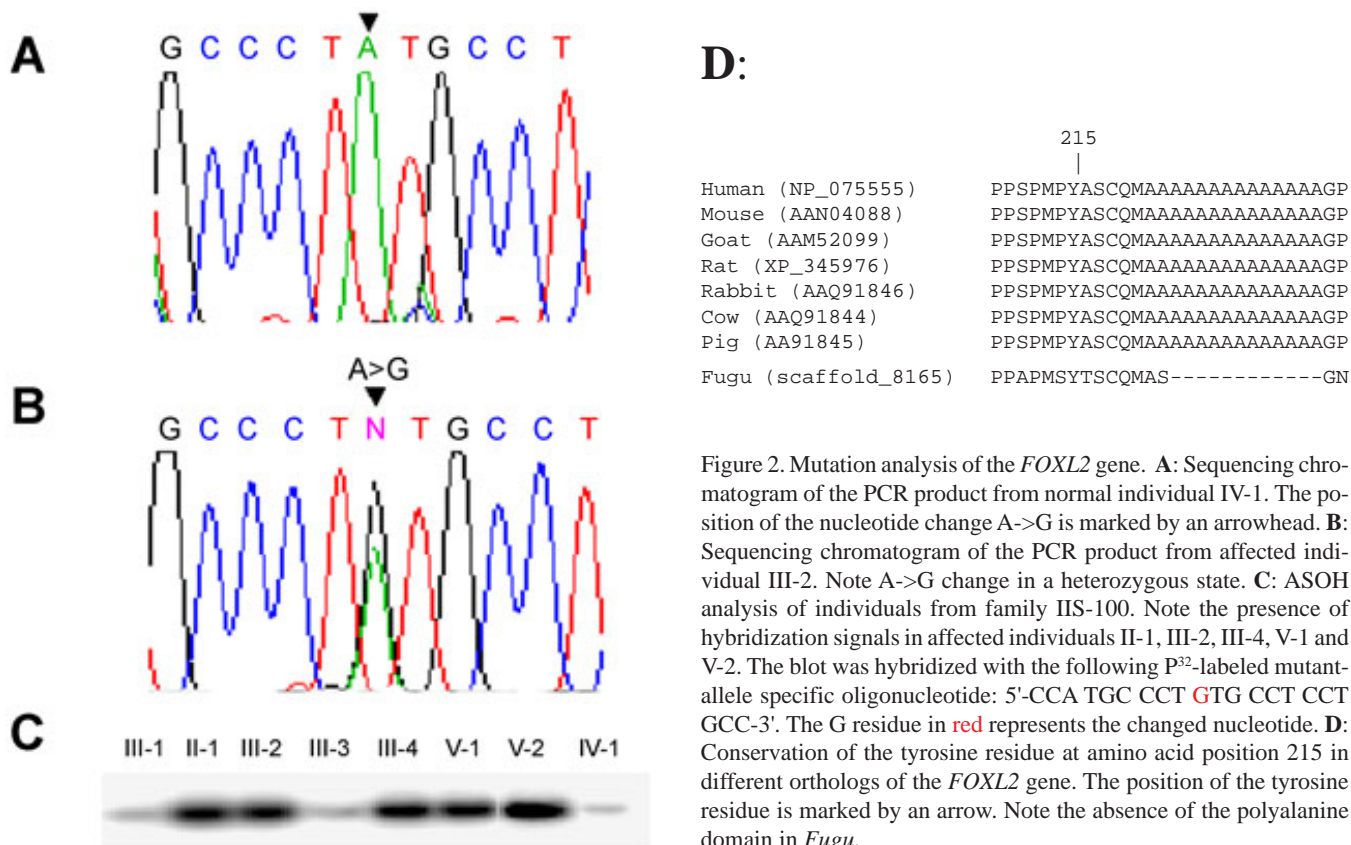


Figure 2. Mutation analysis of the *FOXL2* gene. **A:** Sequencing chromatogram of the PCR product from normal individual IV-1. The position of the nucleotide change A->G is marked by an arrowhead. **B:** Sequencing chromatogram of the PCR product from affected individual III-2. Note A->G change in a heterozygous state. **C:** ASOH analysis of individuals from family IIS-100. Note the presence of hybridization signals in affected individuals II-1, III-2, III-4, V-1 and V-2. The blot was hybridized with the following P³²-labeled mutant-allele specific oligonucleotide: 5'-CCA TGC CCT GTG CCT CCT GCC-3'. The G residue in red represents the changed nucleotide. **D:** Conservation of the tyrosine residue at amino acid position 215 in different orthologs of the *FOXL2* gene. The position of the tyrosine residue is marked by an arrow. Note the absence of the polyalanine domain in *Fugu*.

it is difficult at present to envision the exact effects these mutations will have on the structure and function of the FOXL2 protein. However, it seems that this region is functionally important. These missense changes could act as loss-of-function mutations by decreasing transcriptional control, which is known to be strictly regulated in those rare developmental genetic disorders in which the involvement of other *FOX* genes have been demonstrated [10]. Missense mutations could produce null alleles and the phenotype might be due to haploinsufficiency. It is also possible that the mutant alleles with missense mutations could lead to a BPES phenotype due to a dominant-negative effect. However, all these possibilities remain to be investigated.

In the present family (IIS-100), two female patients (V-1 and V-2; Figure 1A) are pre-pubertal, so we are not able to determine the type of BPES in this family. The same was the case with family f17 with missense mutation S217F reported by De Baere et al. [10] as both daughters were pre-pubertal. Families with two other missense mutations H104R and L106F occurring in the forkhead domain had only male patients [1,17]. In family G-29 with missense mutation N109K occurring in the forkhead domain, there is a known fertile female [17]. A French BPES family with missense mutation I84S occurring in the forkhead domain reported by Dollfus et al. [19] has several females who are known to be infertile. Given this scenario, we are unable to predict at present if both pre-pubertal females in family IIS-100 are going to be fertile (BPES type II) or infertile (BPES type I). This prediction is important for genetic counseling of female patients as it may allow prediction of their fertility. This also poses a genetic counseling dilemma as to what to disclose of their eventual future infertility [20]. In contrast, a genotype-phenotype correlation exists between the types of mutations which are predicted to result in a truncated protein either lacking or containing the forkhead domain and BPES type I or mutations which are predicted to result in an extended protein and BPES type II [10,17]. Phenotypic overlaps do exist between BPES type I and BPES type II [9,20]. In summary, we have reported a novel missense mutation c.881A->G (p.Y215C) in a five generation Indian family with BPES.

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