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Mutations in *FOXL2* Underlying BPES (Types 1 and 2) in Colombian Families

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We report the genetic characterization of one family with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) type 1 and two families with BPES type 2 from a historically isolated population in northwest Colombia. Linkage and haplotype analyses indicate that BPES in these families is linked to 3q23. Mutation screening of *FOXL2* in the family with BPES type 1 revealed a novel 394C → T nonsense mutation which deletes the forkhead DNA binding domain. The two families with BPES type 2 both carry an in-frame 30 bp duplication that leads to the elongation of a polyalanine tract. This duplication has been previously reported in Europe, where recurrent mutation has been demonstrated in unrelated familial and sporadic BPES cases. The recurrent nature of this duplication seems to relate to the secondary structure of this DNA region. The genotype–phenotype correlation seen in the Colombian families is consistent with the recent proposal that BPES type 1 is caused by truncating mutations leading to haploinsufficiency, while BPES type 2 is due to mutations generating elongated protein products.

KEY WORDS: blepharophimosis; forkhead

INTRODUCTION

Blepharophimosis-ptosis-epicanthus syndrome (BPES, OMIM 110100) is a rare disorder characterized by short palpebral fissures, ptosis of eyelids, inverted inner canthal fold between upper and lower lids, and lateral displacement of the inner canthi [Oley and Baraiser, 1988]. Most cases of BPES are sporadic but familial BPES shows autosomal dominant inheritance. Zlotogora et al. [1983] recognized two subtypes of BPES: type 1 in which the syndrome is transmitted only through males (due to female premature ovarian failure), and type 2 with transmission by both affected females and males. Incomplete penetrance has been documented for type 2 BPES [Zlotogora et al., 1983; Temple et al., 1989].

Fukushima et al. [1991] found linkage of BPES to 3q23 and several reports of chromosomal rearrangements, including interstitial deletions and de novo balanced translocations, confirmed this finding [Chandler et al., 1997; Noda et al., 1998]. Fine-mapping of the 3q23 region has led to the recent identification of a gene termed *FOXL2* which is mutated in BPES patients [Crisponi et al., 2001; De Baere et al., 2001]. *FOXL2* is a member of the winged-forkhead family of transcription factors and its mouse homolog is expressed mainly in ovarian follicular cells and the developing eyelids [Crisponi et al., 2001]. Other than 3q23, a second locus for BPES has been mapped to 7p13-p21 in a large Indian family [Maw et al., 1996].

Here we report linkage analysis and mutation screening of three multiplex families with BPES identified in a historically isolated population from Colombia. These families carry microsatellite haplotypes at 3q23 which cosegregate with BPES. Two families carry a previously reported 30 bp duplication and have a BPES type II phenotype. The third family has clinical features of BPES type I and carries a novel *FOXL2* transition 394C → T, resulting in deletion of the forkhead DNA binding domain.

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PATIENTS AND METHODS

Patients

BPES families were ascertained through an index case identified in Medellin, Colombia. Clinical and cytogenetic features of family BPES-2 were previously reported [Ramírez-Castro et al., 1989; Botero, 1998]. A blood sample was collected from individuals consenting to participate in this study and genomic DNA isolated by standard procedures.

High-Resolution Karyotyping

Phytohemagglutinin-stimulated human peripheral blood lymphocytes were grown for 72 h at 37°C in 10 ml RPMI medium supplemented with 20% fetal calf serum and antibiotic. High-resolution banding was performed using standard protocols [Latos-Bieleska et al., 1987]. Lymphocyte cultures were synchronized and blocked in the S-phase by addition of amethopterin (final concentration 10^{-7} M) for 17 h, washed twice with RPMI, and resuspended in fresh medium. Cultures were continued for 5.5 and 6.5 h in the presence of BrdUrd (10 μg/ml). Conventional RBG banding was carried out within 1–2 days [ISCN, 1995].

Genetic Marker Typing

Genotypes of four STR markers in the 3q22-23 region were obtained by PCR using fluorescent methods on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCRs were performed in a final volume of 15 μL with 50–100 ng of genomic DNA, 0.1 μM each primer, 0.2 mM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.45 U Taq DNA polymerase (Promega, Madison, WI). Cycling conditions were: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 48–58°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min.

Linkage Analysis

Two-point linkage analysis was performed using the linkage package assuming autosomal dominant inheritance, a penetrance for BPES of 96.5% [Lathrop et al., 1985], and a mutant allele frequency of 0.0001. Marker

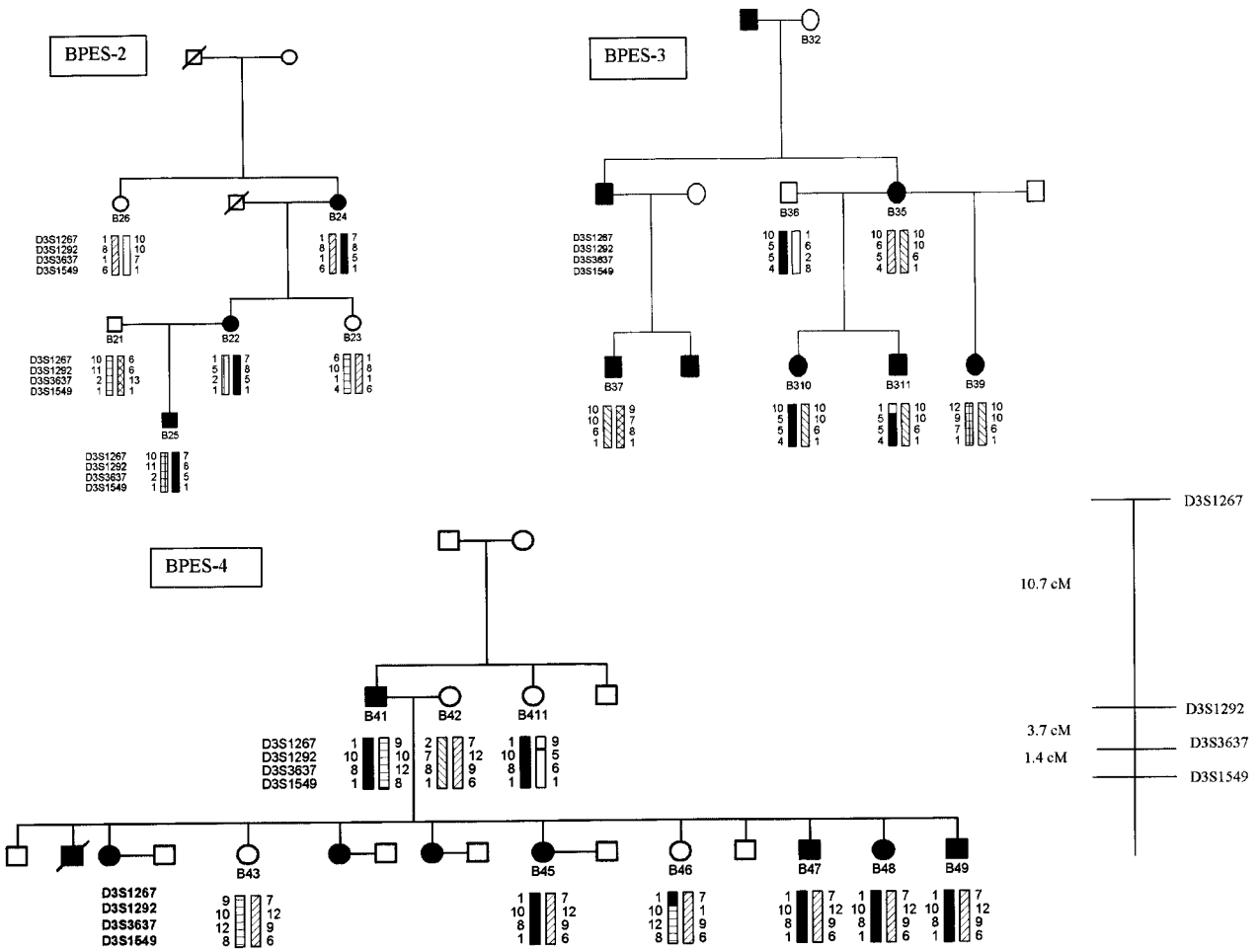


Fig. 1. Colombian families with BPES included in this study and haplotypes obtained at the markers examined in the 3q23 region. Numbers under symbols refer to individual identifiers used in Figure 2.

TABLE I. Two-Point Lod Scores for the 3q23 Markers Examined in Three Colombian BPES Families

		Recombination Fraction								
Marker	Family	0	0.01	0.02	0.1	0.2	0.3	0.4	Zmax	Theta max
D3S1267	BPES-4	−0.55	−0.41	0.76	0.1	0.23	0.18	0.07	0.23	0.21
	BPES-3	0.28	0.27	0.26	0.18	0.1	0.04	0.01	0.28	0
	BPES-2	0.8	0.78	−0.31	0.59	0.38	0.2	0.08	0.8	0
	Total	0.54	0.63	0.7	0.87	0.71	0.43	0.15	0.87	0.09
D3S1292	BPES-4	0.04	0.04	0.03	0.02	0.01	0.01	0	0.3	0
	BPES-3	1.04	1.01	1	0.8	0.55	0.3	0.09	1.04	0
	BPES-2	0.33	0.33	0.32	0.28	0.22	0.15	0.08	0.33	0
	Total	1.41	1.38	1.35	1.1	0.78	0.46	0.17	1.41	0
D3S3637	BPES-4	0.96	0.97	0.98	0.96	0.78	0.52	0.2	0.99	0.04
	BPES-3	1.12	1.1	1.07	0.87	0.6	0.33	0.1	1.12	0
	BPES-2	0.87	0.85	0.83	0.66	0.44	0.25	0.1	0.87	0
	Total	2.96	2.92	2.88	2.48	1.83	1.1	0.4	2.96	0
D3S1549	BPES-4	1.27	1.25	1.24	1.09	0.84	0.54	0.2	1.26	0
	BPES-3	0.95	0.93	0.9	0.73	0.5	0.27	0.08	0.95	0
	BPES-2	0.07	0.07	0.07	0.07	0.05	0.03	0.01	0.07	0.06
	Total	2.28	2.25	2.21	1.89	1.39	0.84	0.29	2.28	0

allele frequencies were obtained from 50 unrelated normal controls from the city of Medellin.

FOXL2 Mutation Screening

DNA fragments covering the coding region of the intron-less *FOXL2* gene were amplified using the primers and PCR conditions reported by Crisponi et al. [2001]. PCR fragments were directly sequenced using the Bid Dye Ready Reaction Sequencing kit on an ABI-373 (Applied Biosystems).

RESULTS

Clinical and Cytogenetic Features

Twenty-two affected individuals were identified in the three Colombian families, of which 13 were available for genetic analyses: three in BPES-2, five in BPES-3, and five in BPES-4 (Fig. 1). All the affected patients in four families showed the characteristic clinical features of BPES. In pedigrees BPES-2 and BPES-3, there are several instances of female transmission of the syndrome and thus correspond to BPES type 2. In family BPES-4, transmission of BPES is confirmed only for one male (B41). In this family the five affected females were of childbearing age (range 23–37 years). Four of these females have been married for 3–17 years without having offspring despite no contraception. Three of the five affected females have irregular menstrual cycles; the other two have normal cycles. Although it was not possible to perform confirmatory hormonal or more invasive investigations, family BPES-2 was given a clinical diagnosis of BPES type 1. High-resolution karyotypes were normal in affected individuals from the three pedigrees.

Haplotype and Linkage Analysis

Each of the Colombian pedigrees shows cosegregation of a 3q23 haplotype and BPES, with each family segregating a different disease haplotype (Fig. 1). In this pedigree set a single recombination event was evidenced between markers D3S1267 and D3S1292 in individual B311. Two-point lod scores (Table I) are consistent with BPES being linked to 3q23 and approach statistical significance for marker D3S3637 (lod score of 2.96 at zero recombination fraction in the combined pedigree set).

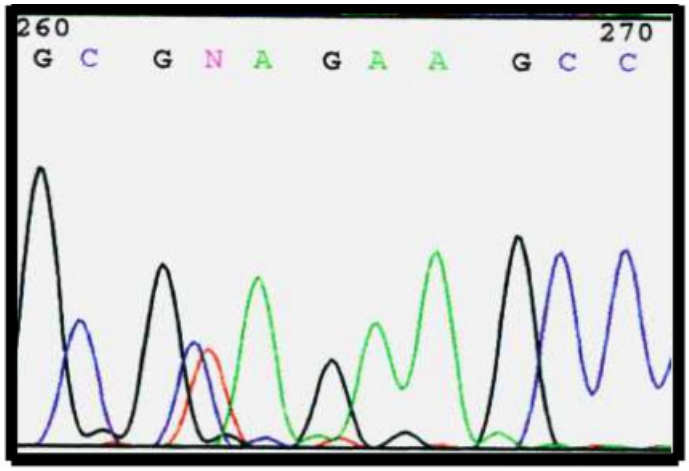
Mutation Screening

Sequencing of *FOXL2* showed that affected individuals from family BPES-4 carry a 394C → T transition which cosegregates with BPES and is not observed in 50 control individuals (mutation screening was facilitated by the fact that the 934C → T mutation leads to the loss of an *HhaI* restriction site). This mutation results in the creation of a stop codon at position 53 (Q53X) of FOXL2. All affected individuals of families BPES-2 and BPES-3 showed a duplication of 30 bp (909–938dup), which was absent in unaffected individuals and in 50 controls (Fig. 2).

DISCUSSION

This is the first molecular study of BPES in a Hispanic population. Patients were ascertained in Antioquia, a region in northwest Colombia with a history of isolation. Recent genetic studies have shown that this population was founded mostly by Spanish men and Native American women [Carvajal-Carmona et al., 2000]. The historical isolation of Antioquia is consistent with the observation of founder effects both for autosomal dominant and recessive disorders in this population [Lendon et al., 1997; Pineda et al., 2001] and with the similarity of mtDNA haplotypes between Antioquia and American Indians currently living in the province [Carvajal-Carmona et al., 2000].

Since two loci are known to be involved in BPES, our first aim was to assess if BPES in the Antioquian



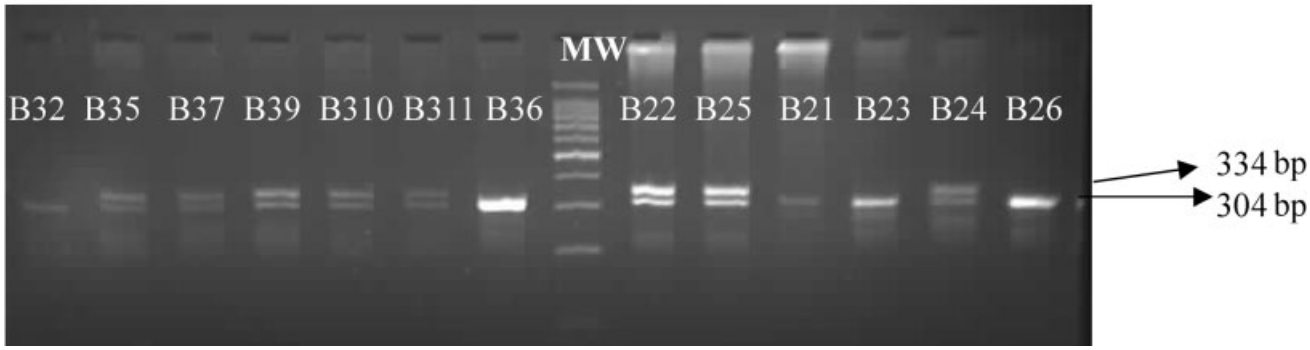


Fig. 2. Mutations in the *FOXL2* gene detected in Colombian BPES families. **A:** Partial sequence output for an affected individual from BPES-4. The C (blue) to T (red) transition at position 394 corresponds to the heterozygous nucleotide (read as N); fourth from the left. **B:** Amplification of a PCR fragment containing the 30-bp insertion in individuals from families BPES-2 and BPES-3. Individual identifiers are those of Figure 1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pedigrees is linked to the 3q23 region. The results shown in Figure 1 and Table I confirm that this is the case, although the presence of a different disease haplotype in each family suggested underlying allelic heterogeneity. Sequence analysis of *FOXL2* revealed the presence of two different mutations in these three families: a novel 394C → T transition in BPES-4 and a previously reported duplication of 30 bp (909–938dup) in BPES-2 and BPES-3. Detailed inquiries have not revealed any genealogical connection between the last two pedigrees, which originate in different areas of the province, suggesting that they might represent a founder effect or independent mutations. This needs to be clarified by typing additional markers in 3q23. The multiple occurrence of this 30-bp duplication has been previously documented in studies of familial and unrelated de novo BPES cases [Crisponi et al., 2001; De Baere et al., 2001]. The mutability of this region of the *FOXL2* gene might relate to the secondary structure of the DNA strands, as the primary sequence forms an almost perfect palindrome flanked by two direct repeats, possibly facilitating duplication during replication [De Baere et al., 2001].

The new mutation identified here (394C → T) results in truncation of the FOXL2 protein at amino acid residue 52 (Q53X), while the 909–938dup is in frame and results in an increase in length of the polyalanine tract located between amino acid residues 221 and 234 of FOXL2. Interestingly, the family that carries the 394C → T mutation (BPES-4) presents infertility, while the two families carrying the 909–938dup both present BPES type 2 (Fig. 1). These observations are consistent with the proposal of De Baere et al. [2001] that truncated FOXL2 causes BPES type 1 while longer FOXL2 proteins lead to BPES type 2. This is the second reported observation of a mutation leading to the loss of the DNA binding domain (forkhead) from FOXL2. This reinforces the notion that the mechanism of dominance leading to BPES type 1 is haploinsufficiency rather than a dominant negative effect. In the case of the 909–938dup, it is unclear if dominance is due to haploinsufficiency or to a dominant negative effect.

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