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Analysis of *FOXL2* detects three novel mutations and an atypical phenotype of blepharophimosis-ptosis-epicanthus inversus syndrome

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ABSTRACT

Background: Mutations in *FOXL2* are known to cause autosomal dominant blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), variably associated with premature ovarian failure. In this study we report results of mutational screening in a Czech and Slovak patient population with BPES.

Design: Case series.

Participants: 13 probands of Czech and 1 proband of Slovak origin with BPES and their available family members.

Methods: Sanger sequencing and multiplex ligation-dependent probe amplification in 14 probands with BPES. Targeted mutational screening in first degree relatives.

Main Outcome Measures: Genetic characterization and phenotype evaluation in Czech and Slovak individuals with BPES and their family members.

Results: Eight different mutations were detected including three novel ones; c.5T>G; p.(Met2Arg), c.197C>A; p.(Ala66Glu) and c.701_702insTGCAGCCGCAGCGGCTGCAGCAGCTGCGGCTGCAGCCGC; p.(Ala222_Ala234dup). In one family, the molecular genetic cause of disease was not identified by the methodology used. In 13 pedigrees, a negative family history suggested a *de novo* origin, which could be confirmed by targeted mutational screening in four families. One 62- year-old female with the c.663_692dup30 mutation had an atypical phenotype presenting as moderate ptosis compensated by frontalis muscle contraction, no epicanthus inversus, and no premature ovarian failure.

Conclusions: The *de novo* mutation rate in *FOXL2* is exceptionally high compared to other dominant disorders manifesting with an ocular phenotype. In cases reporting a negative family history, careful examination of both parents is important to exclude mild features of the BPES phenotype.

Key words: *FOXL2*, blepharophimosis-ptosis-epicanthus inversus syndrome, phenotype

INTRODUCTION

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES, OMIM #110100) is a rare autosomal dominant disorder. Typical features of BPES involve blepharophimosis (vertical and horizontal narrowing of the palpebral fissures), epicanthus inversus (skin fold running from the lower eyelid, inward and upward at the medial canthus), bilateral symmetric ptosis of the upper eyelids, and lateral displacement of the inner canthi.[1,2] Based on the presence or absence of premature ovarian failure in affected females, two forms have been distinguished; type I associated with loss of ovarian function prior to 40 years of age, and type II with eyelid defects as an isolated entity.[1] BPES is caused by mutations within the forkhead box L2 (*FOXL2*, OMIM *605597), currently the only gene associated with this disorder.[3,4] Due to a relatively high rate of *de novo* mutations, detection of sporadic cases is quite common.[5]

This study aimed to identify the molecular basis for BPES in 13 families of Czech, and one family of Slovak origin.

METHODS

The research was reviewed by appropriate ethics committees and adhered to the Declaration of Helsinki, and was approved by the Institutional review board. All photographs that could lead to the patient identification are published with specific consent to do so.

Probands referred with BPES, and their available family members, were recruited following informed consent, and a peripheral venous blood sample collected for molecular genetic investigation. DNA was extracted using a Gentra Puregene DNA Isolation Kit™ (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The entire coding region of *FOXL2* was amplified with three primer sets (primer sequences are available on request). Bidirectional direct sequencing of PCR products was performed using BigDye chemistry on the ABI 3100 Genetic Analyzer following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Generated sequence reads were aligned to the NCBI Reference Sequence

NM_023067.3. Mutation description followed current recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).[6]
Samples negative for mutations in *FOXL2* by Sanger sequencing were further examined for the identification of exonic deletions or duplications by the multiplex ligation-dependent probe amplification (MLPA) method, using the P054-FOXL2-TWIST1 MLPA kit (MRC Holland, Amsterdam, The Netherlands) on the ABI 3130 Genetic Analyzer. Trace data were analyzed using the GeneMapper® Software Version 4.0 (Applied Biosystems).
Potential pathogenicity of detected sequence variants was assessed by determination of segregation with the disease phenotype within the individual families, and mining their frequency in public databases: The Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org), and NHLBI Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/) (both accessed 7 December 2015). Missense changes were scored for disease effect using a range of prediction tools; PROVEAN,[7] SNPs&GO,[8] MutPred,[9] SIFT,[10] PolyPhen-2[11] and MutationTaster.[12] Evolutionary amino acid conservation of affected residues was visualized by multiple sequence alignment using T-Coffee.[13]

RESULTS

Molecular genetic analysis

Of the 14 probands investigated, 13 were Czech and one was of Slovak Caucasian ancestry (proband 4). Rare sequence *FOXL2* variants considered pathogenic were found in 13 of these index cases (Table 1). All identified mutations were present in a heterozygous state. No consanguinity was reported in any of the families. None of the mutations, including the two novel missense changes, were present in publicly available databases of human variation (ExAC and EVS showing frequency data from more than 60,000 individuals), supporting their causative role in disease development.
The mutations detected included three missense changes, of which c.5T>G; p.(Met2Arg) and c.197C>A; p.(Ala66Glu) (Supplementary Fig. 1) were observed in association with BPES for the first time. Unfortunately, the affected son of proband 1

was unavailable for testing to confirm segregation of c.5T>G with the disease-phenotype. Most prediction tools evaluating possible pathogenicity of missense variants and high conservation of the affected amino acid across species supported the disease-causing role of the three missense mutations identified (Supplementary Table 1 and Supplementary Fig. 2).

The clinically unaffected father of proband 2 exhibited lower peaks for c.197C>A on both forward and reverse sequence chromatograms. Multiple sequencing reactions (n=4) were undertaken with genomic DNA extracted from leucocytes, with persistence of this change on all chromatograms, suggesting mosaicism (Fig. 1). The third missense mutation c.644A>G; p.(Tyr215Cys) has been previously observed fully segregating with the BPES phenotype in a multiple generation family.[14]

Duplications leading to polyalanine expansions were found in six families, of which c.701_702insTGCAGCCGCAGCGGCTGCAGCAGCTGCGGCTGCAGCCGC; p.(Ala222_Ala234dup) was novel at the DNA level (Supplementary Fig. 1). Two probands had a duplication predicted to cause a frameshift and subsequent creation of a premature stop codon p.(Pro287Argfs*75), while one patient exhibited a deletion leading to a frameshift that abolishes the wild type termination codon p.(Pro317Glyfs*214). Deletion of the entire open reading frame was observed in one family.

In one family, Sanger sequencing and MLPA testing was unable to identify a pathogenic mutation. In only one pedigree there was an extensive family history of the disease, consistent with autosomal dominant inheritance. In all other families, the absence of clinical BPES in preceding generations suggested a *de novo* origin of the mutation (Supplementary Fig. 3). This was confirmed in two probands by negative mutational screening in parental DNA. In the rest of the families, DNA samples were not available to confirm the presumed *de novo* nature.

Genotype-phenotype correlations

Based on the referral information from collaborating clinicians, all probands exhibited the typical ocular phenotype of BPES with ptosis, blepharophimosis and epicanthus inversus apparent from birth. No additional ocular features occasionally reported in

association with BPES, i.e. nystagmus, microphthalmos, microcornea, and stenosis of the lateral canaliculi, nor any developmental abnormalities, were observed.[4,15]
One relative, (mother of proband 4) aged 62 years, heterozygous for c.663_692dup30 in *FOXL2*, was not aware of being affected with BPES until our investigation. She was documented to have high myopia and astigmatism. After bilateral cataract surgery, performed at the age of 62, her BCVA was 0.5 in both eyes. Detailed inspection of her face in photographs from childhood, and subsequent ophthalmological assessment, showed a longstanding bilateral symmetrical ptosis with compensatory usage of musculus frontalis, but no evidence of epicanthus inversus (Fig. 2). Her inner intercanthal distance was 31 mm (normal range for Caucasian females 32.95 ± 2.90 mm). The horizontal palpebral fissure length measured 21 mm bilaterally (normal range 29.40 ± 2.46 mm), and palpebral fissure height was decreased to 7 mm bilaterally (normal range 10.65 ± 1.21 mm).[16] She reported menopause occurring after the age of 40, indicating BPES type II.

DISCUSSION

This study is the first to molecularly characterize BPES syndrome in probands from the Czech and Slovak Republic, with *FOXL2* mutations detected in 13/14 probands (93%). Three novel mutations were identified, in addition to an atypical phenotype present in one family member.
Missense changes are an infrequent cause of BPES, representing only 11% of intragenic mutations.[4] In the current study the proportion of missense changes was slightly higher at 23%. This ratio may be however influenced by the relatively small sample size. Both novel missense sequence variants identified; c.5T>G; p.(Met2Arg) and c.197C>A; p.(Ala66Glu), were regarded as pathogenic or possibly pathogenic based on *in silico* analysis including an absence in variation databases. The c.644A>G; p.(Tyr215Cys) has been previously reported in association with BPES.[14] The father of proband 2, who had no clinical evidence of BPES, is thought to represent a case of possible somatic mosaicism. Analysis of repeated sequence chromatograms showed approximately one third mutation peak heights for the

mutated allele (c.197C>T) on both forward and reverse strands Unfortunately, additional tissue was not available for further evaluation.

In five probands we identified known polyalanine expansions leading to p.(Ala225_Ala234dup). In proband 9, a previously unreported change at the DNA level c.701_702insTGCAGCCGCAGCGGCTGCAGCAGCTGCGGCTGCAGCCGC was observed, however is predicted to lead to a known protein effect; p.(Ala222_Ala234dup). Polyalanine expansions are usually fully penetrant for the eyelid phenotype.[17,18] The absence of the typical epicanthus inversus appearance in the mother of proband 4 carrying the p.(Ala225_Ala234dup) is therefore a rare finding. Ophthalmic examination also showed that she had reduced BCVA most likely due to stimulus deprivation amblyopia, present in approximately 41% of BPES patients.[19] The mild phenotype observed highlights the necessity of careful examination.

FOXL2 open reading frame deletions are found in 10% of patients with typical BPES [5], but was present in only one proband (7%) in this study. In one family (7%), with an affected mother and her daughter, we failed to identify a disease causing mutation, however assessment of possible deletions in regulatory regions, estimated to represent 5% of molecular defects in BPES,[5] or non-coding mutations in general was not undertaken in this analysis.

Knowledge of the BPES type is important for genetic counselling because of the association of female infertility due to premature ovarian failure, characterized by absent menarche, or premature depletion of ovarian follicles before the age of 40 years.[20] Predictions based solely on the genotype are however uncertain as some mutations have been described in association with both phenotypes. Most of the affected female probands in this study were at the prepubertal developmental stage, so their BPES type could not be assessed. Proband 11 had the c.843_859dup17 mutation, expected to lead to a truncated protein p.(Pro287Argfs*75), and suffered from primary amenorrhea, indicating BPES type I. We were also able to determine the age of menopause onset (45 years) in the affected mother of proband 4, and can thus confirm the previously reported association of BPES type II with p.(Ala225_Ala234dup). Deletion of the entire open reading frame leading to haploinsufficiency has been associated with ovarian dysfunction.[21] The sporadic 1.5-

year-old female proband with this pathogenic variant will therefore require close endocrine and gynaecological follow-up.

The *de novo* rate of mutations in *FOXL2* is high suggesting hypermutability of the gene.[22] Targeted mutational screening in clinically unaffected parents of cases 5, 7 and 13 confirmed the first occurrence in the affected proband. Based on family history, a likely *de novo* origin was present in nine other families. In family 2 the father of the proband was a possible mosaic for the pathogenic variants, our study therefore suggests that clinically unaffected individuals may carry a somatic mutation acquired during early development. In cases reporting a negative family history, careful examination of both parents is important as they may be undiagnosed, manifesting only with a milder phenotype of BPES.

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REFERENCES

1. De Baere E, Copelli S, Caburet S, Laissue P, Beysen D, Christin-Maitre S, Bouchard P, Veitia R, Fellous M. Premature ovarian failure and forkhead transcription factor FOXL2: blepharophimosis-ptosis-epicanthus inversus syndrome and ovarian dysfunction. *Pediatr Endocrinol Rev* 2005; **2**: 653-60.
2. Oley C, Baraitser M. Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES syndrome). *J Med Genet* 1988; **25**: 47-51.
3. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat Genet* 2001; **27**: 159-66.

4. Verdin H, De Baere E. FOXL2 impairment in human disease. *Horm Res Paediatr* 2012; **77**: 2-11.
5. Beysen D, De Paepe A, De Baere E. FOXL2 mutations and genomic rearrangements in BPES. *Hum Mutat* 2009; **30**: 158-69.
6. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 2000; **15**: 7-12.
7. Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of amino acid substitutions and indels. *PLoS One* 2012; **7**: e46688.
8. Calabrese R, Capriotti E, Fariselli P, Martelli PL, Casadio R. Functional annotations improve the predictive score of human disease-related mutations in proteins. *Hum Mutat* 2009; **30**: 1237-44.
9. Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, Mooney SD, Radivojac P. Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics* 2009; **25**: 2744-50.
10. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009; **4**: 1073-81.
11. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods* 2010; **7**: 248-9.
12. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods* 2010; **7**: 575-6.
13. Di Tommaso P, Moretti S, Xenarios I, Orobitg M, Montanyola A, Chang JM, Taly JF, Notredame C. T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res* 2011; **39**: W13-7.
14. Kumar A, Babu M, Raghunath A, Venkatesh CP. Genetic analysis of a five generation Indian family with BPES: a novel missense mutation (p.Y215C). *Mol Vis* 2004; **10**: 445-9.

15. Cai T, Tagle DA, Xia X, Yu P, He XX, Li LY, Xia JH. A novel case of unilateral blepharophimosis syndrome and mental retardation associated with de novo trisomy for chromosome 3q. *J Med Genet* 1997; **34**: 772-6.
16. Barretto RL, Mathog RH. Orbital measurement in black and white populations. *Laryngoscope* 1999; **109**: 1051-4.
17. De Baere E, Dixon MJ, Small KW, Jabs EW, Leroy BP, Devriendt K, Gillerot Y, Mortier G, Meire F, Van Maldergem L, Courtens W, Hjalgrim H, Huang S, Liebaers I, Van Regemorter N, Touraine P, Praphanphoj V, Verloes A, Udar N, Yellore V, Chalukya M, Yelchits S, De Paepe A, Kuttenn F, Fellous M, Veitia R, Messiaen L. Spectrum of FOXL2 gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES) families demonstrates a genotype--phenotype correlation. *Hum Mol Genet* 2001; **10**: 1591-600.
18. De Baere E, Beysen D, Oley C, Lorenz B, Cocquet J, De Sutter P, Devriendt K, Dixon M, Fellous M, Fryns JP, Garza A, Jonsrud C, Koivisto PA, Krause A, Leroy BP, Meire F, Plomp A, Van Maldergem L, De Paepe A, Veitia R, Messiaen L. FOXL2 and BPES: mutational hotspots, phenotypic variability, and revision of the genotype-phenotype correlation. *Am J Hum Genet* 2003; **72**: 478-87.
19. Dawson EL, Hardy TG, Collin JR, Lee JP. The incidence of strabismus and refractive error in patients with blepharophimosis, ptosis and epicanthus inversus syndrome (BPES). *Strabismus* 2003; **11**: 173-7.
20. Beck-Peccoz P, Persani L. Premature ovarian failure. *Orphanet J Rare Dis* 2006; **1**: 9.
21. Beysen D, Raes J, Leroy BP, Lucassen A, Yates JR, Clayton-Smith J, Ilyina H, Brooks SS, Christin-Maitre S, Fellous M, Fryns JP, Kim JR, Lapunzina P, Lemyre E, Meire F, Messiaen LM, Oley C, Splitt M, Thomson J, Van de Peer Y, Veitia RA, De Paepe A, De Baere E. Deletions involving long-range conserved nongenic sequences upstream and downstream of FOXL2 as a novel disease-causing mechanism in blepharophimosis syndrome. *Am J Hum Genet* 2005; **77**: 205-18.
22. Beysen D, Moumne L, Veitia R, Peters H, Leroy BP, De Paepe A, De Baere E. Missense mutations in the forkhead domain of FOXL2 lead to subcellular

mislocalization, protein aggregation and impaired transactivation. *Hum Mol Genet* 2008; **17**: 2030-8.

TABLE

Table 1: List of mutations detected in *FOXL2* by Sanger sequencing in a cohort of Czech and Slovak probands with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES). Mutation description was based on the Reference Sequence NM_023067.3. All sequence variants were present in a heterozygous state. BPES type was established only in family 4 with an affected postmenopausal female. Pedigrees of probands 1-5, 7-14 can be viewed in Supplementary Fig. 3.

No	Gender / Age	Family history/ relatives known to be affected	Mutation	Protein consequence	Relatives tested	MLPA	Other information
1	M/36 y	Y/son	c.5T>G	p.(Met2Arg)	Mother and brother – negative	Negative	Suggestive of a *de novo* origin
2	M/3 y	N	c.197C>A	p.(Ala66Glu)	Mother – negative, father – mosaicism suspect, paternal grandparents negative	NP	*De novo* origin- suggestive of somatic mosaicism in an asymptomatic parent
3	M/38 y	Y/father, sister, daughter	c.644A>G	p.(Tyr215Cys)	Relatives not available	Negative	Suggestive of a *de novo* origin in the proband's father
4	M/35 y	Y/mother, brother, son, nephew	c.663_692dup30	p.(Ala225_Ala234dup)	Mother - positive	NP	Suggestive of a *de novo* origin in the probands mother BPES type II
5	F/6 m	N	c.663_692dup30	p.(Ala225_Ala234dup)	Parents - negative	NP	*De novo* origin
6	M/30 y	Y/ several family members from 5 generations	c.672_701dup30	p.(Ala225_Ala234dup)	Relatives not available	NP	BPES reported in multiple generations
7	M/11 m	N	c.672_701dup30	p.(Ala225_Ala234du	Parents - negative	NP	*De novo* origin

				p)			
8	F/1 m	N	c.672_701dup30	p.(Ala225_Ala234dup)	Relatives not available	NP	Suggestive of a *de novo* origin
9	M/3 y	Y/father	c.701_702insTGCAGCCGCAGCGGCTGCAGCAGCTGCGGCTGCAGCCGC	p.(Ala222_Ala234dup)	Father - positive	NP	Suggestive of a *de novo* origin in the proband's father
10	M/48 y	N	c.843_859dup17	p.(Pro287Argfs*75)	Relatives not available	NP	Suggestive of a *de novo* origin
11	F/16 y	N	c.843_859dup17	p.(Pro287Argfs*75)	Relatives not available	NP	Suggestive of a *de novo* origin Primary amenorrhea
12	M/33 y	Y/son	c.948_955del8	p.(Pro317Glyfs*214)	Relatives not available	NP	Suggestive of a *de novo* origin
13	F/18 m	N	ORF deletion	p.0?	Parents - negative	Positive	*De novo* origin Normal psychomotor development
14	F/1 y	Y/mother	Not identified	p.?	Mutation not identified	Negative	Suggestive of a *de novo* origin in the proband's mother

M = male, F = female, y = years, m = months, Y = yes, N = no, MLPA = Multiplex ligation-dependent probe amplification, NP = not performed, ORF = open reading frame

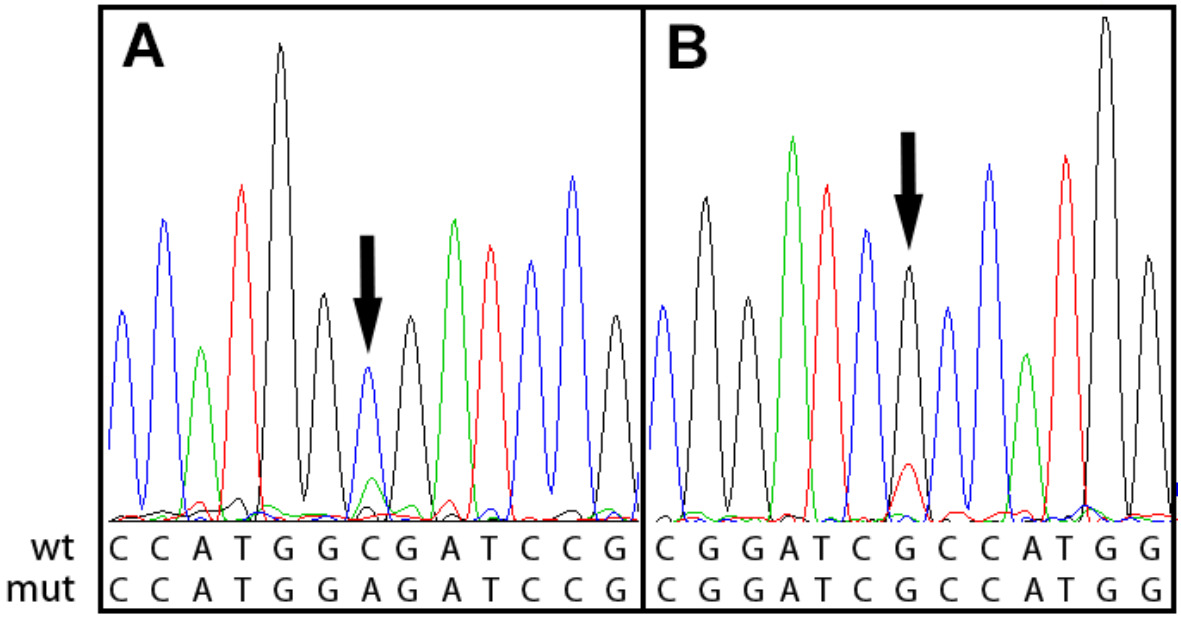

Figure 1: Sequence chromatograms in the clinically unaffected father of proband 2 harbouring c.197C>A; p.(Ala66Glu) in *FOXL2*. Unequal peak height observed on both forward (A) and reverse strands (B) (arrows), on repeated testing is suggestive of mosaicism. Wt indicates the wild type and mut the mutated sequence.

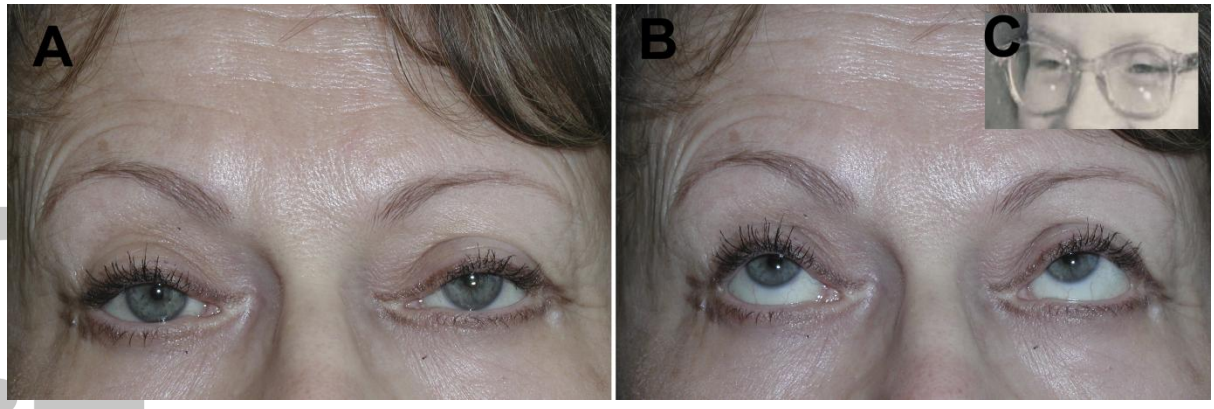

Figure 2: An atypical phenotype in a female with p.(Ala225_Ala234dup) mutation in *FOXL2*. Age 62 years: Absence of typical epicanthus inversus and telecanthus, with elevation of the normal medial canthal position, associated with the presence of blepharophimosis and mild ptosis, causing a narrowing of the vertical palpebral fissure. This is compensated for by increased frontalis muscle action to elevate the eyebrows and lids, primary position (A) and upward gaze (B). Age 10 years: photograph documenting bilateral ptosis (C).