Clinical and genetic studies of 17 Han Chinese pedigrees and 31 sporadic patients with blepharophimosis-ptosis-epicanthus inversus syndrome

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Purpose: To investigate the molecular pathogenesis of a large group of Han Chinese patients with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), and to evaluate the correlation between the phenotype and genotype for these patients.

Methods: Seventy-six affected individuals, including 45 patients from 17 pedigrees and 31 sporadic patients, were recruited with their family members. All participants underwent complete clinical examinations and were classified as having type I or II based on whether they had premature ovarian failure. The patients' genomic DNA was extracted. A genetic test was performed with direct sequencing of the coding regions of the *forkhead transcriptional factor 2* (*FOXL2*) gene. Variations were analyzed using online databases and programs. Genotype–phenotype correction was investigated.

Results: Seventy-six affected and 75 unaffected individuals underwent clinical evaluations and genetic testing. Only one family was diagnosed with type I; the others could not be classified because of a lack of female patients or a definite history of premature ovarian failure. Twenty-seven variations were identified, including 12 novel and 15 previously reported variations. Six variations were detected repeatedly in different nonconsanguineous pedigrees. Four indel variations, located in the alanine/proline-rich region of the *FOXL2* gene, presented with a relatively higher frequency. Two rare double variations were detected in two sporadic patients. *FOXL2* gene variations were not detected in five sporadic patients. The phenotype varied among different families and patients, although they carried the same variations.

Conclusions: We identified 12 novel variations in the *FOXL2* gene that would expand the spectrum of the *FOXL2* variation database. In addition, we found that the alanine/proline-rich region is a variation hotspot in the *FOXL2* gene. The genotype–phenotype correlation is not easy to establish due to clinical and genetic heterogeneity.

Blepharophimosis-ptosis-epicanthus syndrome (BPES; OMIM 110100) is a complex eyelid disorder characterized by blepharophimosis, ptosis, epicanthus inversus, and telecanthus [1,2], with an estimated prevalence of 1 in 50,000 individuals in the world [3]. This disorder may be categorized into two subtypes by the presence (type I) or absence (type II) of premature ovarian failure (POF) [4].

BPES is inherited in an autosomal dominant pattern and is rarely reported as an autosomal recessive trait [5]. Mutations in the *forkhead transcriptional factor 2* (*FOXL2*, OMIM 605597) gene have been identified as the underlying molecular pathogenesis of BPES [6,7]. To date, more than 200 mutations in *FOXL2* have been reported in patients with type I and type II BPES [3,8-10].

FOXL2 is located on chromosome 3q22.3 and encodes a 376-amino-acid protein by a single exon. The N-terminus of the FOXL2 protein contains a 100-amino-acid DNA-binding forkhead domain, while its carboxyl terminus contains an alanine/proline-rich region with a poly-alanine (poly-Ala) tract highly conserved in mammals [11,12]. The FOXL2 protein is highly expressed in the mesenchyme of the developing eyelids, periocular muscles, granulosa cells in the ovary, and gonadotropic cells in the anterior pituitary. As a transcriptional regulator, FOXL2 is involved in ovary differentiation and maintenance, repressing the genetic program of somatic testis determination.

Previous studies have suggested that the mutant types of FOXL2 could be related to the phenotypes of BPES. For example, mutations resulting in a truncated protein before the poly-alanine tract could be associated with BPES type I, whereas mutations resulting in an extended protein could result in BPES type II [13,14]. However, because of the genetic and clinical heterogeneity of BPES manifestations, there is no solid evidence to support the correlation between genotype and phenotype.

In this study, we analyzed the clinical features and investigated mutations in the *FOXL2* gene of 17 Han Chinese

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pedigrees and 31 sporadic patients with BPES. In addition, we evaluated the correlation between the BPES phenotype and genotype in these patients.

METHODS

Participants: A total of 151 participants were retrospectively recruited from 48 Han Chinese families; probands from 17 pedigrees and 31 sporadic patients were included with their family members. All participants underwent physical examinations and were asked for their family and medical history. BPES diagnoses were made using the following clinical criteria: blepharophimosis, ptosis, epicanthus inversus, and telecanthus. POF was confirmed from medical records and defined as cessation of menses for a duration of ≥6 months at the age <40 years and a concentration of follicle-stimulating hormone (FSH) of >40 IU/l [15]. The inheritance pattern was confirmed by the family history. The study was approved by the Beijing Children's Hospital Ethics Committee and conducted in accordance with the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects. Informed consent was obtained from all participants or their guardians for research according to the Guidance of Sample Collection of Human Genetic Diseases through the Ministry of Public Health of China.

Variation screening: Three milliliters peripheral venous blood was collected from each participant. Genomic DNA was extracted from each individual's lymphocytes according to the standard protocol (Roche Biochemical, Inc, Palo Alto, CA) and stored in a -80 °C refrigerator for use. Amplification of the genomic fragments encompassing *FOXL2* coding regions (NCBI human genome build 35.1, NC_000003 for gDNA, NM_023067 for mRNA, and NP_075555 for protein) was performed with PCR using the following primer pairs: FOXL2-F: 5′- GAG CTC AAG CTT CCA TGA TGG CCA GCT ACC CCG AG-3′, FOXL2-R: 5′- GGT GGA TCC TCA GAG ATC GAG GCG CGA ATG C-3′. PCR was performed with 100 ng genomic DNA, 5 μl of 10× KOD buffer, 4 μl a deoxy-ribonucleoside triphosphate (dNTP) mixture, 0.8 μl of Kod DNA polymerase (TOYOBO Co., Ltd, Osaka, JPN), 2 μl of each primer, 3 μl of dimethyl sulfoxide (DMSO) and double distilled water (ddH$_2$O) to a final volume of 50 μl. PCR amplification was performed initially at 94 °C for 2 min, followed by 30 cycles at 96 °C for 15 s, 63 °C for 15 s, 68 °C for 1 min, with 0.3 °C increased each cycle, and finally an elongation step at 68 °C for 1 min. The PCR products were separated on a 2% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Bidirectional sequencing was performed using the BigDye

Terminator Cycle Sequencing V3.1 kit on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems PerkinElmer, Foster City, CA).

Sequence analysis: Sequencing results were assembled and analyzed using the SeqMan program of DNASTAR software (DNASTAR Inc., Madison, WI). The variations were searched in databases, including dbSNP151, EXAC, gnomAD 2.1, ClinVar, and HGMD2021. Pathogenicity prediction scores were obtained for missense variants using SIFT, PolyPhen-2, and CADD, and further confirmed with 3D construction of the protein using the PyMOL program. Variations were named following the nomenclature recommended by the Human Genomic Variation Society (HGVS). The pathogenicity of the variations was assessed according to the American College of Medical Genetics and Genomics/ Association for Molecular Pathology (ACMG/AMP) guidelines. The gene–disease relationship was evaluated through the ClinGen Gene Curation Framework with a calculated score of ≥12 to be pathogenetic (Appendix 1).

RESULTS

Clinical evaluations: Seventy-six individuals were diagnosed with BPES. The ratio of men to women was 40 to 36. An autosomal dominant inheritance mode was confirmed because BPES presented vertically from generation to generation in 17 pedigrees regardless of gender, and all patients were heterozygous variation carriers. Only one family was diagnosed with BPES type I, as all affected women showed oligomenorrhea and irregular menses with two cases of infertility. In this family, the proband's mother had aborted six times. In addition, many women in this family were diagnosed with POF based on endocrine testing and ultrasonic examinations. Seven families were recognized as having BPES type II based on a normal menstrual history and delivery history in the affected women. Nine of the 17 pedigrees could not be categorized into any groups due to a lack of female patients or a definite history of premature ovarian failure. Detailed clinical information is listed in Appendix 2.

Variation analysis: Screening of the *FOXL2* gene showed that 27 different variations were detected in individuals with BPES, including 12 novel and 15 previously reported nucleotide variations (Figure 1). Pathogenic variations were detected in all 17 pedigrees and 26 sporadic patients, apart from five sporadic patients who were not found to carry pathogenic variations. Because some variations were present in two or more nonconsanguineous pedigrees or in sporadic patients, only 27 kinds of mutations were detected. Of the variations, ten were single nucleotide changes, and 17 were indel variations. Three single nucleotide variations were novel identified,

including one nonsense variation, c.214G>T (p. Glu72Ter*), and two missense variations, c.150C>G (p. Asp50Glu) and c.326A>T (p. Asn109Ile), that were predicted to be deleterious to the protein function and structure through online program analysis by using SIFT, PolyPhen-2, and CADD and the PyMOL program (Table 1 and Figure 2). Interestingly, these two novel missense variations were detected as a double variation in a sporadic patient. Seven previously reported single nucleotide variations were detected from eight pedigrees, including two nonsense variations, c.157C>T (p.Gln53*) and c.244C>T(p.Gln82*), and five missense variations, c.307C>T (p.Arg103Cys), c.331T>C(p.Cys111Arg), c. 650C>T (p. Ser217Phe), c.644A>G (p.Tyr215Cys), and c.1045C>G (p.Arg349Gly). As the second double variation detected in this study, the variation c.1045C>G (p. Arg349Gly) was detected with the known variation c.855_871dup (p.

His291Argfs*71) in a patient who inherited a missense variation, c.1045C>G (p. Arg349Gly), from her father, while the variation c.855_871dup (p. His291Argfs*71) was a de novo variation.

Nine novel indel variations were detected, including c.54delA (p. Glu19Argfs*131), c.411_412del(p. Met137Ilefs*101), c.533_542del (p. Val178Alafs*90), c.547delG (p. Ala183profs*88), c.675_690del (p. Ala226Leufs*40), c.684delA (p. Ala229Leufs*43), c.708delC (p. Gly237Valfs*34), c.965_978del (p. Leu321Profs), and c.993_995delinsA (p. Pro333Glyfs). The nine novel variations and eight previously reported variations are listed in Appendix 2. Four previously reported indel mutations recurred in the pedigrees and sporadic patients with a relatively high frequency, including c.663_692dup (p. Ala225-Ala234dup),
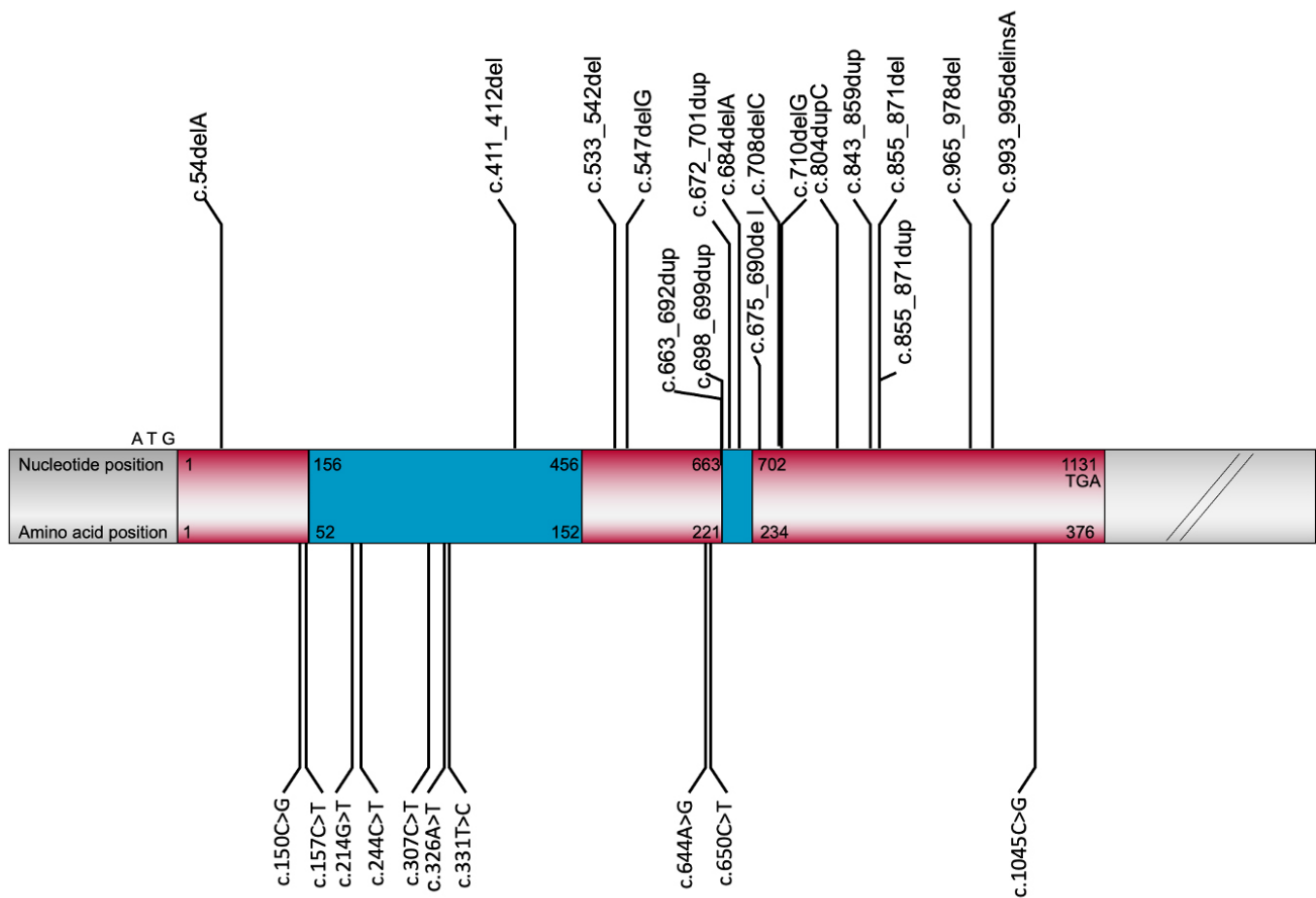

Figure 1. Summary of the *FOXL2* variations in this study. Boxes with different colors indicate the forkhead domain and the poly-Ala tract (blue) and the link structures (red). Variants of deletions, insertions, and duplications are labeled above the box, and missense and nonsense variations are labeled below.

TABLE 1. DESCRIPTION OF SIFT, POLYPHEN2, CADD SCORES AND PYMOL STRUCTURE PREDICTION FOR 2 NOVEL MISSENSE VARIATIONS.

Mutation	Protein	SIFT	Polyphen2	CADD	PyMol
150C>G	Asp50Glu	0.056	0.994	24	Unstable structure due to a wild-type Asp replaced by a mutant type Glu with a larger molecular weight than Asp; and caused by loss of interaction between Asp and Lys at codon 48 due to amino acid replacement.
326A>T	Asn109Ile	0	1.000	28	Unstable structure caused by a hydrophilic polar amino acid of Asn replaced by a hydrophobic nonpolar amino acid of Ile.

c.672_701dup (p. Ala225-Ala234dup), c.843_859dup (p. Pro287Argfs*75), and c.855_871dup (p. His291Argfs*71), which were detected in the alanine/proline-rich region. To examine the possibility of a common origin among families carrying the same variation, four single nucleotide polymorphisms (SNPs), rs7432551, rs11924939, rs61750361, and rs1405762888, in and around *FOXL2* were genotyped in the patients. Haplotype analysis indicated that the variations c.663_692dup (p. Ala225-Ala234dup), c.672_701dup (p. Ala225-Ala234dup), c.843_859dup (p. Pro287Argfs*75), and c.855_871dup (p. His291Argfs*71) occurred independently in each family. These four indel variations presented a total of 20 times in all patients carrying mutations in the *FOXL2* gene, accounting for 44% (20/45) of all detected variations. The variations c.663_692dup (p. Ala225-Ala234dup) and c.672_701dup (p. Ala225-Ala234dup) were predicted to result in poly-alanine expansions, while the variations c.843_859dup (p. Pro287Argfs*75) and c.855_871dup (p. His291Argfs*71) would produce a truncated protein. The variation c.698_699dup (p. Ala228-Ala232dup) was reported previously as a homozygous variation but detected in one of the families as a heterozygous variation. All variations mentioned above were considered disease-causing variations evaluated by ACMG/AMP and the Gene Curation Framework. Detailed curation summaries and ACMG/AMP classification are listed in Appendix 1 and Appendix 2.

DISCUSSION

In this study, we analyzed the clinical characteristics of a large cohort of patients with BPES and investigated variations in their *FOXL2* gene. We found 27 different variations in the *FOXL2* gene in these patients, including 12 novel mutations.

Previous studies have shown that intragenic variations account for more than 70% of all *FOXL2* variations, including frameshift, in-frame, nonsense, and missense variations [13,14]. In addition, more than 10% of *FOXL2* variations have been detected outside the coding region, located either upstream or downstream of the coding region [16], which is usually neglected and may result in a negative result in genetic testing. In this study, *FOXL2* variations were detected in approximately 93% (71/76) of the patients, while only five patients were not found to have *FOXL2* variations. Further studies are being conducted to investigate the disease-related variations outside the open reading frame of the *FOXL2* gene for these five patients.

We reviewed all intragenic variations in *FOXL2* reported in the ClinVar database and analyzed them with the 27 different pathogenic variations detected in this study. We found that about 19% of the variations occur in the alanine/proline-rich region, where 92% are indel variations. In terms of the present data, eight of the 27 (30%) variations were detected in the alanine/proline-rich region, and all are indel variations. In addition, the fact that variants of
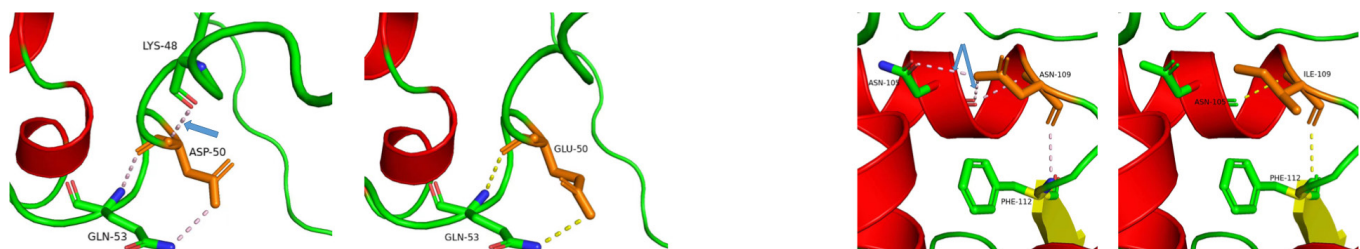

Figure 2. PyMOL structure prediction for two novel missense variations. The variation c.150C>G, p. (Asp50Glu) is shown on the left, and c.326A>T, p. (Asn109Ile) is shown on the right. The blue arrows point to where the connections between amino acids have changed.
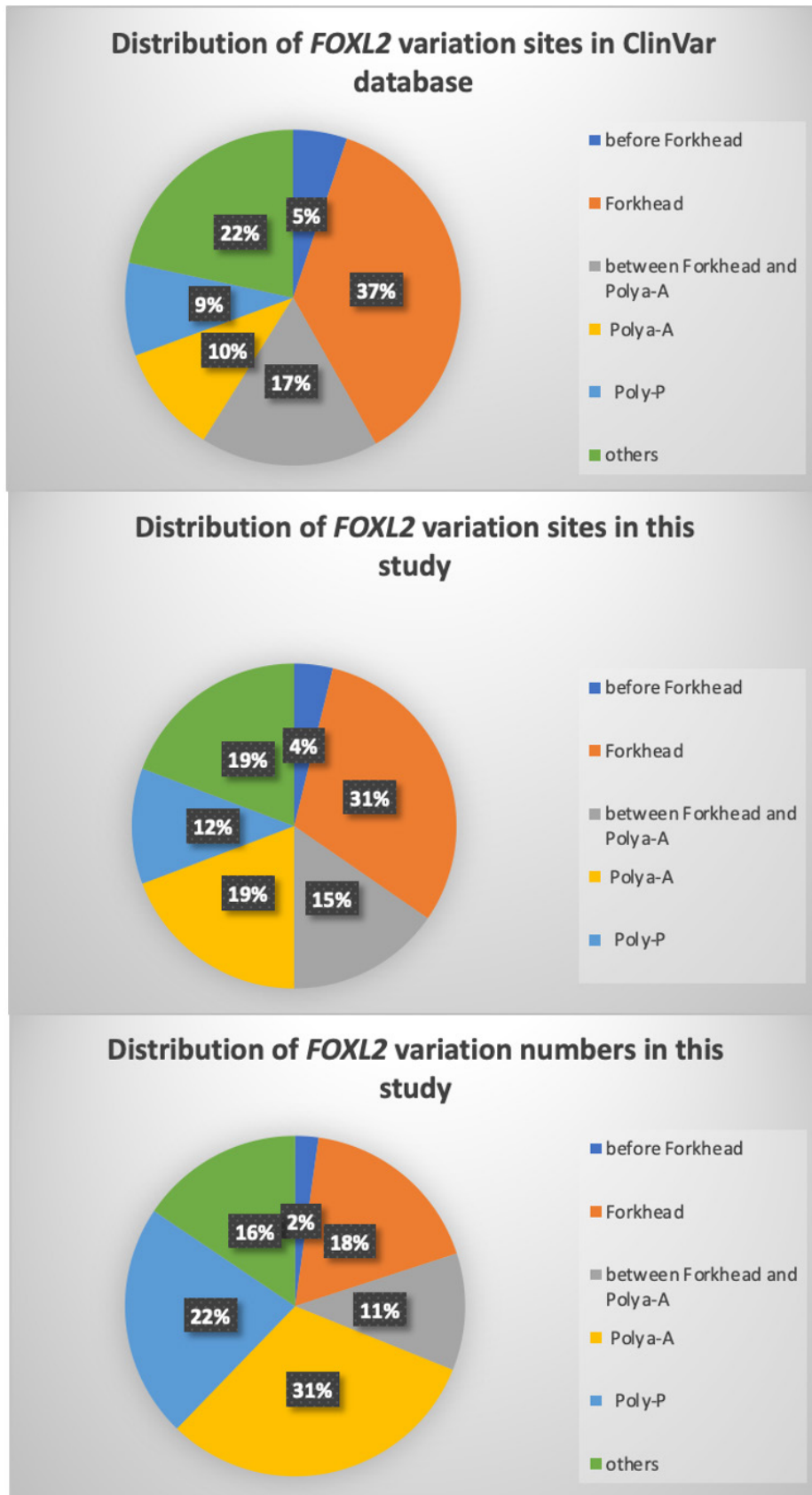
Figure 3. Distribution of *FOXL2* variations.

c.663_692dup (p. Ala225-Ala234dup), c.672_701dup (p. Ala225-Ala234dup), c.843_859dup (p. Pro287Argfs*75), and c.855_871dup (p. His291Argfs*71) occur more than once in the alanine/proline-rich region illustrates that it is a mutation hotspot region (Figure 3).

The alanine/proline-rich region plays an important role in localizing the FOXL2 protein in the nucleus. These indel variations would change the alternative polyadenylation (APA) events, affect the fate of the gene transcript, including its half-life and cellular localization, and impair normal protein folding, resulting in aggregate formation in the affected tissues, which is the pathogenesis of poly-alanine expansion disorder. Recently, more than 40 diseases have been associated with repeat expansion disorders that mainly affect the nervous and/or muscular system and include myotonic dystrophies, Huntington's disease, and fragile X syndrome [17,18].

The variation c.698_699dup (p. Ala228-Ala232dup) was detected as a homozygous variation in a consanguineous Indian family in which only the individuals who were homozygous carriers presented as having BPES, while heterozygous carriers did not have symptoms, which showed an autosomal recessive inheritance pattern [5]. However, this variation was detected as a heterozygote in the patient in the present study, with typical features of BPES. As mentioned above, BPES is mainly inherited in an autosomal dominant pattern, but in rare cases it can be inherited in an autosomal recessive mode. A previous study showed that BPES could be caused by either haploinsufficiency or a dominant-negative effect [19]. However, the details of the molecular mechanism are not clear. A mutation of c.698_699dup (p. Ala228-Ala232dup) would be predicted to produce a truncated protein containing a complete forkhead domain. On one hand, the possibility of the dominant-negative effect cannot be excluded in the heterozygous variation because the truncated protein may still be able to bind to DNA. On the other, haplotype insufficiency could be the more likely mechanism of homozygous variation because the heterozygous carrier did not have symptoms. Similarly, the heterozygous variations c.650C>T (p. Ser217Phe) and c.1045C>G (p. Arg349Gly) could be likely to result in haplo-insufficiency or a dominant-negative effect because the carriers with these variations present either as normal individuals or a BPES patient. Further studies are necessary to explore the exact molecular mechanism for these variants.

We also investigated the genotype–phenotype correlation in patients with *FOXL2* variations. Previous studies showed that BPES type I was associated with variations ahead of the poly-alanine tract, while type II was associated with variations that result in poly-Ala expansion [13,14]. However, we did not find a genotype–phenotype correlation in the present study patients because only one family with type I was identified. In addition, the previously reported mutation of c.672_701dup (p. Ala225-Ala234dup), which was predicted to result in poly-Ala expansion, was shown to result in type I and type II in the present study patients. Therefore, we believe that it is not comprehensive to classify patients into type I or type II from the perspective of genotype alone.

In summary, we investigated the molecular pathogenesis for a large cohort of patients with BPES and found 12 novel mutations in the *FOXL2* gene, which would further expand the spectrum of *FOXL2* variations, and would be helpful for future research on the molecular pathogenesis. In addition, we explored the genotype–phenotype correlation for the study patients with BPES and found heterogeneity in the clinical and genetic characteristics. Although we did not establish a correlation between genotype and phenotype for the present study's patients with BPES, these data will contribute to further research on BPES.

APPENDIX 1. THE GENE–DISEASE RELATIONSHIP EVALUATED THROUGH THE CLINGEN GENE CURATION FRAMEWORK.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. THE GENOTYPE AND PHENOTYPE OF PATIENTS.

To access the data, click or select the words "Appendix 2."

REFERENCES

1. Allen CE, Rubin PA. Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES): clinical manifestation and treatment. Int Ophthalmol Clin 2008; 48:15-23. [PMID: 18427257].

2. Krepelova A, Simandlova M, Vlckova M, Kuthan P, Vincent AL, Liskova P. Analysis of FOXL2 detects three novel mutations and an atypical phenotype of blepharophimosis-ptosis-epicanthus inversus syndrome. Clin Experiment Ophthalmol 2016; 44:757-62. [PMID: 27283035].

3.   Chai P, Li F, Fan J, Jia R, Zhang H, Fan X. Functional Analysis of a Novel FOXL2 Indel Mutation in Chinese Families with Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome Type I. Int J Biol Sci 2017; 13:1019-28. [PMID: 28924383].

4.   Bertho S, Pasquier J, Pan Q, Le Trionnaire G, Bobe J, Postle-thwait JH, Pailhoux E, Schartl M, Herpin A, Guiguen Y. Foxl2 and Its Relatives Are Evolutionary Conserved Players in Gonadal Sex Differentiation. Sex Dev 2016; 10:111-29. [PMID: 27441599].

5.   Nallathambi J, Moumne L, De Baere E, Beysen D, Usha K, Sundaresan P, Veitia RA. A novel polyalanine expansion in FOXL2: the first evidence for a recessive form of the blepharophimosis syndrome (BPES) associated with ovarian dysfunction. Hum Genet 2007; 121:107-12. [PMID: 17089161].

6.   Yang XW, He WB, Gong F, Li W, Li XR, Zhong CG, Lu GX, Lin G, Du J, Tan YQ. Novel FOXL2 mutations cause blepharophimosis-ptosis-epicanthus inversus syndrome with premature ovarian insufficiency. Mol Genet Genomic Med 2018; 6:261-7. [PMID: 29378385].

7.   Grzechocinska B, Warzecha D, Wypchlo M, Ploski R, Wielgos M. Premature ovarian insufficiency as a variable feature of blepharophimosis, ptosis, and epicanthus inversus syndrome associated with c.223C > T p. (Leu75Phe) FOXL2 mutation: a case report. BMC Med Genet 2019; 20:132-[PMID: 31366388].

8.   Lin B, Zeng B, Zhao J, Xu T, Wang Y, Hu B, Li F, Zhao Q, Liu R, Liu J, Chen JM, Huang D, Wang Y. Seven Novel and Three Known Mutations in FOXL2 in 10 Chinese Families with Blepharophimosis Syndrome. Curr Mol Med 2018; 18:152-9. [PMID: 30198434].

9.   Jiang H, Huang X, Su Z, Rao L, Wu S, Zhang T, Li K, Quan Q, Zhang K. Genetic analysis of the forkhead transcriptional factor 2 gene in three Chinese families with blepharophimosis syndrome. Mol Vis 2013; 19:418-23. [PMID: 23441113].

10. Bunyan DJ, Thomas NS. Screening of a large cohort of blepharophimosis, ptosis, and epicanthus inversus syndrome patients reveals a very strong paternal inheritance bias and a wide spectrum of novel FOXL2 mutations. Eur J Med Genet 2019; 62:103668-[PMID: 31077882].

11. Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, De Baere E, Messiaen L, Cotinot C, Fellous M, Veitia RA. Evolution and expression of FOXL2. J Med Genet 2002; 39:916-21. [PMID: 12471206].

12. Verdin H, De Baere E. FOXL2 impairment in human disease. Horm Res Paediatr 2012; 77:2-11. [PMID: 22248822].

13. 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. [PMID: 11468277].

14. 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. [PMID: 12529855].

15. Anasti JN. Premature ovarian failure: an update. Fertil Steril 1998; 70:1-15. [PMID: 9660412].

16. Beysen D, De Paepe A, De Baere E. FOXL2 mutations and genomic rearrangements in BPES. Hum Mutat 2009; 30:158-69. [PMID: 18726931].

17. Pisarska MD, Bae J, Klein C, Hsueh AJ. Forkhead l2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. Endocrinology 2004; 145:3424-33. [PMID: 15059956].

18. Davies JE, Rubinsztein DC. Polyalanine and polyserine frame-shift products in Huntington's disease. J Med Genet 2006; 43:893-6. [PMID: 16801344].

19. 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. [PMID: 11175783].