· Basic Research ·

Identification and functional analyses of a novel *FOXL2* pathogenic variant causing blepharophimosis, ptosis, and epicanthus inversus syndrome

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Abstract

● **AIM:** To discover the molecular pathogenic basis of the blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES), and to predict the clinical subtype according to *in vitro* experiments, which is significant to the prognosis.

● **METHODS:** A 3-year-old sporadic female patient with typical clinical manifestations of BPES was enrolled. The coding region of forkhead box L2 (*FOXL2*) gene was sequenced, and the functional assays were performed *in vitro* by Western blotting, subcellular localization experiment, luciferase reporter assay, and quantitative real-time polymerase chain reaction.

● **RESULTS:** A novel *FOXL2* point pathogenic variant (c.274G>T) was detected, resulting in a truncated protein (p.E92*). Functional studies demonstrated that the *FOXL2* pathogenic variant induced the subcellular mislocalization and the abnormal transcriptional activity on promoters of the steroidogenic acute regulatory protein (*StAR* or *STARD1*) gene and the odd-skipped related 2 transcription factor (*OSR2*) gene.

● **CONCLUSION:** A novel pathogenic variant is identified to expand the spectrum of the known *FOXL2* mutations. The *in vitro* experiments provide reference data and more insights to the molecular pathogenesis of BPES. The predicted high risk of ovarian insufficiency makes it significant for the patient enrolled to have further follow-up and therapy concerning female endocrinology.

● **KEYWORDS:** novel mutation; pathogenic variant; blepharophimosis-ptosis-epicanthus inversus syndrome; *FOXL2*

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INTRODUCTION

Blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES; the Online Mendelian Inheritance in Men #110100), first reported by Ammon in 1841, is a rare congenital disease, whose prevalence is estimated as 1/50 000 according to statistics[1]. Depending on the clinical features, BPES is separated into two main phenotypes: the type I patients have premature ovarian insufficiency/failure (POI/POF) and eyelid malformations at the same time while the type II patients only have deformities in eyelids[2].

The forkhead box L2 (*FOXL2*) gene, encodes a conserved transcription factor, which is a member of the winged helix transcription factor family. *FOXL2* contains one forkhead domain, serving to affect the promoters of downstream genes, and the poly-Ala tracts which were associated with the intracellular localization[3]. *FOXL2* is the only known gene responsible for BPES[4].

FOXL2 involves in a lot of physiological processes serving as a transcription factor[5-6]. During the development of an embryo, *FOXL2* selectively expresses in the mesenchymal stem cell of the fetal ovary and developing eyelids, which has been proven in animal models. The *FOXL2* knockout mice exhibit absent upper eyelid and failure of somatic cell development in the fetal ovary[7]. Besides the embryogenetic stage, researchers have proven that FOXL2 is involved in almost all functions of the adult ovary[8]. The promoter of the steroidogenic acute regulatory protein (*StAR* or *STARD1*) gene has been proven to be the direct target of *FOXL2*[9], which plays a significant role in granulosa cells. The regulatory interaction between *FOXL2* and the odd-skipped related 2 transcription factor (*OSR2*) gene is crucial in the development of facial bones and eyelids, which has been proven by previous studies[10].

Female patients with BPES are usually in childhood when visiting hospitals, without symptom available in favor of

forecasting the POI/POF, and the variation of ovarian reserve function make it difficult to make a prompt diagnosis[11]. Accordingly, researching the genotype-phenotype correlation is of great value for predicting the classification of BPES. De Baere *et al*[12] subdivide the *FOXL2* mutations into nine classes, based on the predicted protein; Moumné *et al*[13] once reported that the *FOXL2* mutations expressing truncated proteins upstream of the poly-Ala tract likely lead to type I BPES, while type II BPES usually caused by the *FOXL2* mutation resulting in extended proteins. However, these claims cannot explain all the cases reported, such as the patients with heteroplasmy[14-15], and hence the genotype-phenotype relationship of BPES still needs further researches for providing evidences.

In this study, we performed gene sequencing for *FOXL2* in a sporadic case. The further researches concerning biological functions were carried out to provide follow-up medical advice and clues for determining the genotype-phenotype correlation.

SUBJECTS AND METHODS

Ethical Approval The present study conformed to the Declaration of Helsinki and was approved by the ethics committee of the Plastic Surgery Hospital (Institute), CAMS, PUMC (2023-53). Informed consent was obtained from guardians of the patient.

Patient A 3-year-old girl with eye malformations (Figure 1A) was admitted to the Plastic Surgery Hospital (Institute), CAMS, PUMC seeking surgical treatment. The patient was born at 38wk of gestation after an uncomplicated pregnancy and a normal delivery from a nonconsanguineous healthy Chinese parents both without genetic disorder. Received systematic ophthalmic examinations, the patient was diagnosed with BPES for the classic symptoms of blepharophimosis, ptosis, and epicanthus inversus. Photos were taken before the surgery. The patient was tested with normal 46, XX karyotype, and her ovarian function can not be evaluated because of the age.

DNA Extraction and Sequence The patient's genomic DNA was extracted from the leukocytes of peripheral venous blood using QIAamp Blood DNA Mini kit (Qiagen, Germany) following the manufacturer's instruction. Three sets of premiers were designed to amplify the region of *FOXL2* through polymerase chain reaction (PCR): *FOXL2*-1F: GGTGAGCACAGGAGGACATAA; 1R: GAGCGAGTACCGGCAGATT; *FOXL2*-2F: GTGAGCACAGGAGGACATAA; 2R: AGTTTGAGACTTGGCCGTAA; *FOXL2*-3F: TGCTTCATCAAGGTGCCG; 3R: GCACAAGCGAACTGCAGG. After purifying, the PCR products were sequenced using the Sanger sequencing (Applied Biosystems, CA, USA) to confirm the pathogenic variant. Then, the outcome was analyzed using GeneMapper V3.5, and the nomenclature of the novel pathogenic variant was determined according to Human Genome Variation Society Guidelines. The MutationTaster online software was used for predicting the possible pathogenicity.

Plasmid Construction The coding sequence of the *FOXL2* opening reading frame (ccds3105.1) was cloned into pcDNA3.1 and pEGFP-C1 vectors, producing pcDNA3.1-FOXL2 and pEGFP-FOXL2. Using wild-type *FOXL2* as the template, the mutant plasmids with c.274G>T were constructed by site-directed mutagenesis (QuikChange Lightning Multi Site-Directed Mutagenesis Kit, Agilent), producing pcDNA3.1-FOXL2-G274T and pEGFP-FOXL2-G274T. The promoter and 5'-UTR region of *StAR* and *OSR2* were inserted into the pGL3- Basic to build the luciferase reporter vectors. All the constructs were verified without additional mutations through Sanger sequencing.

Western Blotting The murine leydig tumor cell line 1 (MLTC-1) cells were grown in 6-well plates 24h before the transfection, maintained in the Roswell Park Memorial Institute-1640 (Procell, China) with 10% fetal bovine serum (Procell, China) and 1% P/S at 37°C in a 5% CO_2 atmosphere. Then, the medium was changed into Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA), and the MLTC-1 cells of each dish were transfected with 2.5 μg pEGFP-C1, pEGFP-FOXL2 or pEGFP-FOXL2-G274T using 7.5 μL Lipofectamine 3000 reagent and 5.0 μL P3000 reagent (Invitrogen, USA). Finishing 48h of culture, the cells were washed with phosphate-buffered saline (PBS; Beyotime, Shanghai, China) twice after removing the media. Then, the cells of one well were lysed with 150 μL mixed liquor of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China). The protein concentrations were determined by using the BCA Protein Assay Kit (Beyotime, Shanghai, China). A total of 35 mg protein from each sample added loading buffer were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel, and next the protein was transferred to nitrocellulose filter membranes (Osmonics, USA). After blotted by TBST with 5% skimmed milk powder, membranes were incubated in primary antibodies at 4°C overnight and the corresponding secondary antibodies for 1h at room temperature. Using The ChemiDoc MP system (Bio-Rad Laboratories, California, USA) to detect the immunotagged proteins. The primary antibodies we used are anti-GFP antibody (1:1000, Cat No. 3h9, Proteintech), anti-FOXL2 antibody (1.5 μg/mL, ab5096, Abcam), and anti-GAPDH antibody (1:5000, ab8245, Abcam). Western stripping buffer (Beyotime, Shanghai, China) was used between the chemiluminescent immunoassay of GFP and incubating the anti-GAPDH antibody.

Subcellular Localization Experiment MLTC-1 cells were seeded into the 35 mm confocal dishes and were transfected with pEGFP-C1, pEGFP-FOXL2 or pEGFP-FOXL2-G274T respectively using the same type and dose of reagents with the Western blotting experiment. After 72h of culturing, the nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Germany) after 48h of the transfection, and subcellular localization was observed by Zeiss LSM 800 confocal laser scanning microscopy.

Luciferase Reporter Assay Human embryonic kidney 293 (HEK-293) cells were seeded into 24-well plates 24h before the transfection, maintained in the Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% P/S at 37°C in a 5% CO_2 atmosphere. The 2.0 μL/well of Lipofectamine 2000 reagent (Invitrogen, USA) was used to transfect pcDNA3.1 (vector), pcDNA3.1-FOXL2 (WT), pcDNA3.1-FOXL2-G274T (MT), and the reporter constructs described above. The double luciferase assays of every single reporter plasmid were divided into 6 groups including 1) 500 ng vector; 2) 500 ng WT; 3) 500 ng MT; 4) 250 ng WT plus 250 ng vector; 5) 250 ng MT plus 250 ng vector; 6) 250 ng WT plus 250 ng MT, together with 500 ng luciferase reporter plasmids (either pGL3-StAR or pGL3-OSR2) and 25 ng PRL-TK vectors (Promega) per well. The total DNA content was maintained at 1025 ng/well. The cells were incubated with plasmids in DMEM for 8h, and then the culture was continued for another 48h with the complete media. Following the protocol of Dual-Luciferase Reporter Assay System (Promega, USA), the luciferase intensities were measured by EnSpire Muitilable Plate Reader (PerkinElmer, USA).

Quantitative Real-time PCR HEK-293 cells were plated in 12-well plates and transfected with 1.0 μg of pcDNA3.1-FOXL2, pcDNA3.1-FOXL2-G274T, and empty pcDNA3.1 vector respectively using 2.0 μL of P3000 reagent and 3.0 μL of Lipofectamine 3000 reagent each well. After 48h of culture, total RNA was extracted with TRIzol LS Reagent (Cat No.10296010, Invitrogen) following the operating instructions, and the final RNA concentrations were measured by NanoDrop 2000 (Thermo, USA). cDNA was synthesized using the Universal RT-PCR Kit (M-MLV; Promega, USA). Then, the SYBR Green real-time PCR was performed on LightCycler 96 (Roche). GAPDH gene was chosen for normalization. The primer pairs for qRT-PCR are listed as follows: GAPDH-F: 5'-CAACAGCCTCAAGATCATCAGCA-3'; R: 5'-GTCATGAGTCCTTCCACGATAC-3'; StAR-F: 5'-TTTAGTACCAAGAAAACAGGGATG-3'; R: 5'-CACACCCATATCAGCCACT-3'; FOXL2-F: 5'-GTCCGGCATCTACCAGTACATCA-3'; R: 5'-CCAGCCCTTCTTATTCTTCTCG-3'.

RESULTS

Identification of a Novel Pathogenic Variant in FOXL2 The sequences of PCR products derived from the patient's genomic DNA revealed a novel pathogenic variant (c.274G>T), that resulted in a truncated protein with 92 amino acids (p.E92*) due to the introduction of a premature stop codon (Figure 1B and 1C). The variant was confirmed absent by Sanger sequencing in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP).

As the online software (https://www.mutationtaster.org/) predicted, the truncated protein translated by the *FOXL2* pathogenic variant is only composed of 92 amino acids, without poly-Ala tract and partial FKH domain (the 3rd α-helical structure). The PhyloP and PhastCons scores were 3.821 and 1, which means the pathogenic variant was highly conserved among species.

Effects of the Novel Pathogenic Variant on Subcellular Localization As is shown in the Figure 2A, an approximate 35 kDa band, 8 kDa higher than the EGFP, was observed with the GFP antibody after being transfected with the pEGFP-FOXL2-G274T, which verified that the mutated *FOXL2* could be translated into protein in MLTC-1. An approximate 70kDa protein was detected clearly using either the GFP or FOXL2 antibodies when the MLTC-1 transfected with the pEGFP-FOXL2. However, no 70 kDa band was observed in other tracks, which confirmed that the *FOXL2* expression was absent in MLTC-1 and the protein translated by the mutated *FOXL2* was truncated. For the reason that the antibody of FOXL2 we used here was bound with the C-terminal of wild-type FOXL2. To further investigate the changes in biological functions of this *FOXL2* variant, we conducted the subcellular localization experiment using the MLTC-1 cells. As the Figure 2B exhibits, the wild-type FOXL2 connected with EGFP tag was distributed exclusively in the nucleus region stained with DAPI diffusely, while the mutant FOXL2 can be detected in both the cytoplasm and nucleus.

Effects of the Novel Pathogenic Variant on Transcriptional Activity The results of the reported assays showed that the repression ability of WT protein on *StAR* promoter was obvious, while the MT protein failed to inhibit the luciferase activity of the reporter vectors. The luciferase activity didn't exhibit a decreasing trend as we increase the transfection amount of the MT plasmids (Figure 3A). To simulate the physiological conditions, equal concentrations of MT/WT vectors were transfected testing the dominant negative effect of the novel pathogenic variant. The results showed that the activity of wild-type FOXL2 in binding the promoter of *StAR* did not be diminished when co-transfected with mutant FOXL2 (Figure 3C).

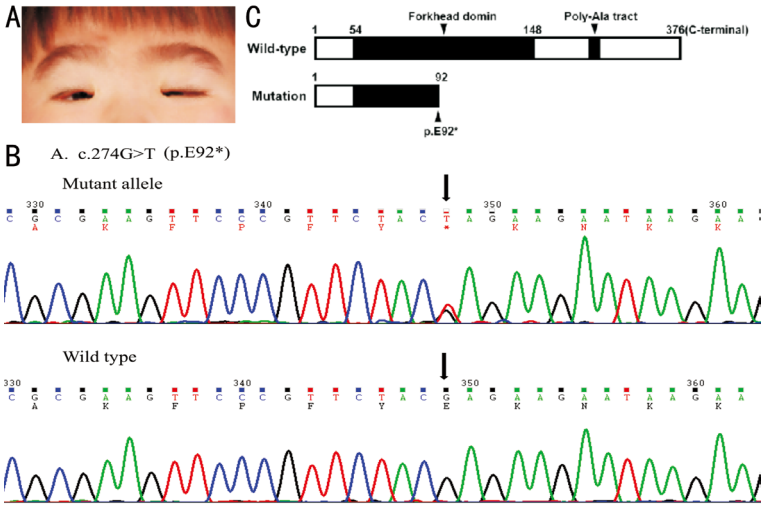

Figure 1 The novel pathogenic variant and its clinical presentation A: Facial photograph of the sporadic patient; B: Sequencing result revealed the novel *FOXL2* pathogenic variant c.274G>T (p.E92*). The arrow indicates the position of the nonsense pathogenic variant. C: Schematic representation of the truncated protein.

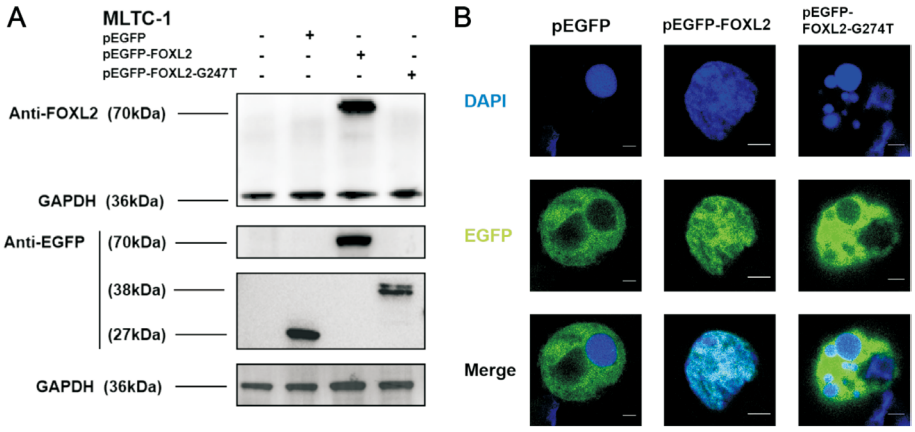

Figure 2 Protein expression and distribution A: Western blot analysis showed the expression of the wild-type FOXL2 and the pathogenic variant FOXL2 with the anti-FOXL2 antibody and the anti-GFP tag antibody. The position of the bands was consistent with the predictions. GAPDH served as the reference gene. B: Subcellular localization of EGFP, FOXL2 and the FOXL2-G274T. The first line shows the nuclear stained with DAPI; the second line exhibits the subcellular localization of EGFP or FOXL2 as the fusion protein tagged with EGFP. The third line contains merged images of the above pictures. Scale bars=5 μm.

We next test the capacity of the mutant FOXL2 in activating the *OSR2* promoter. As is shown in the (Figure 3B), the MT lost the function of activating the *OSR2* promoter for the reason that no statistical difference of luciferase activity was detected between the cells transfected with the empty vectors and the MT plasmids. The WT FOXL2 activated the *OSR2* promoter in a dose-dependent manner. In addition, an obvious dominant negative effect was detected when the cells were co-transfected with the same dose of WT and MT (Figure 3D).

Effects of the Novel Pathogenic Variant on Expressions of Downstream Genes To further confirm the effects exerted by this novel *FOXL2* variant on ovarian functions, we test the endogenous mRNA expressions of *StAR* using the qRT-PCR. The results (Figure 3E) showed that the expression levels of the target gene were similar when transfected with empty pcDNA3.1 vectors and the pcDNA3.1-FOXL2-G274T plasmids. In contrast, the cells transfected with wild-type *FOXL2* exhibited obvious decrease in the expression level of *StAR*. The results of qRT-PCR verified the functional absence of this mutant FOXL2 protein in serving as the repressor of the *StAR*.

DISCUSSION

The FOXL2, serving as a conserved transcription factor, plays an important role in the ovary and eyelid from embryonic stages to adult stages[16]. As for blepharophimosis, ptosis, and epicanthus inversus syndrome, surgical treatments for eye malformations and predicting the occurrence of premature ovarian insufficiency/failure are major demands for clinical work. Many mature operations have been used in the treatments of BPES, which give patients better appearances and achieve a lower incidence of amblyopia among the diseased cohort. However, no feasible and correct laboratory index

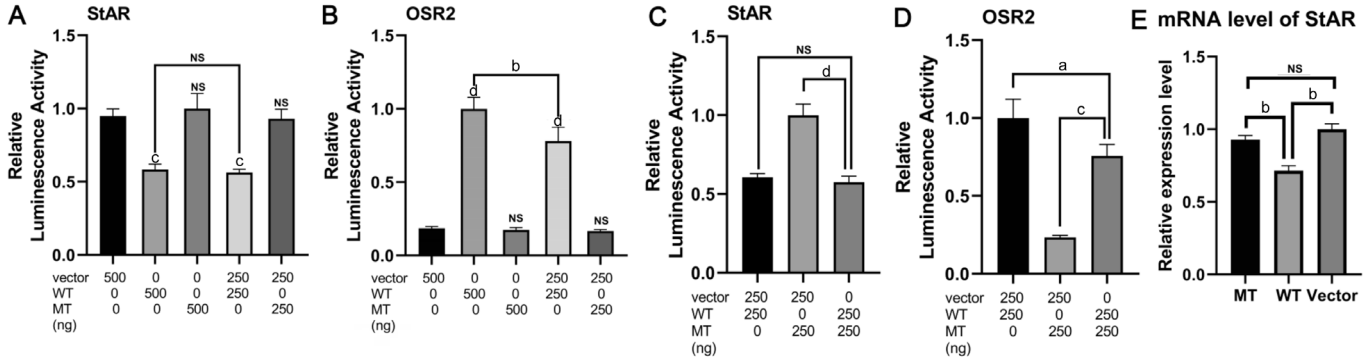

Figure 3 Transcriptional activity of p.E92* mutant FOXL2 The pcDNA3.1 (vector), pcDNA3.1-FOXL2 (WT) or pcDNA3.1-FOXL2-G274T (MT) plasmids were co-transfected with the luciferase vectors driven by the StAR promoter (A) and OSR2 promoter (B). Co-transfected the same dose of WT and MT to detect the potential dominant negative effect of the pathogenic variant on StAR promoter (C) and OSR2 promoter (D). Relative expression level of StAR, measured by qRT-PCR, was compared in the cells transfected with empty vectors, WT and MT plasmids. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001, [d]*P*<0.0001. NS: *P*>0.05.

guiding type I BPES patients in childhood and their family to make preparations, like tissue banking, for the upcoming POI/POF[8]. Support and advice should be offered to relative families. Accordingly, the significance of the genotype-phenotype relationship goes without saying. As is mentioned in the preceding part of this paper, because of the lack of a golden standard, BPES type I and II still cannot be distinguished through simple gene sequencing. For the sporadic cases without family medical histories, the prediction of phenotype is even harder. Hence, research concerning the biological functions of new protein pathogenic variants is still needed to be performed. In this research, a novel *FOXL2* nonsense mutation 274G>T (p.E92*) was reported, located in the forkhead domain. For this 3-year-old female patient, the prediction of the subtype is of great importance for future treatments.

To begin with, the subcellular localizations were tested, for the reason that the wrong location and aggregation could reflect dysfunction of the mutant protein, and the aggregation may predict the dominant negative effec[3,17]. The results of the Western blot ensured the validity of the subcellular localization experiments. In addition, the results verified the FOXL2 pathogenic variant was truncated. As a transcription factor, the FOXL2 was transferred into the nucleus for functioning, which corresponded with the result. In contrast, the mislocalization of the MT protein demonstrated the dysfunction of the mutant *FOXL2*[18-19].

Next, the bioactivities of mutant FOXL2 as a transcription factor were researched by detecting the interactions with the downstream genes[15]. StAR is indispensable in the process of delivering large amounts of cholesterol into the mitochondria, where the cholesterol could switch to pregnenolone[20]. The expression of *StAR* implies the differentiation of granulosa cell. FOXL2 serving as the main passive regulator, could preserve primordial follicles and prevent premature depletion of ovarian follicles by suppressing the promoter of *StAR*[9]. Thus some FOXL2 mutations failing to bind its expression would induce the BPES type I[5,14]. *OSR2* knockout mice exhibited eyelid malformations caused by the growth defects of the palate shelf[21], and the FOXL2 was proven that could activate its promoter directly. The perturbation of this regulatory interaction could contribute to eyelid malformation of the BPES[22]. What's more, *OSR2* was also detected expressing in not only the eyelid dermis but also the uterus of neonatal infants[10]. Thus, we chose these two downstream genes in vitro cell experiments to predict the pathogenicity in ovarian function and periorbital development. The pathogenesis of BPES could attribute to haploinsufficiency, and the dominant negative effect, FOXL2 pathogenic variant obstructing the action of the wild-type protein, which is more pathogenic[23].

The dual-luciferase reporter assay showed that the novel variant lost its biological function as a transcription factor, and less amount of wild-type protein could achieve a complete reaction with the promoter of *StAR*, which could explain the formation of the two subtypes: the development of eyelids is more sensitive to the variation in the quantity of FOXL2. When the cells were co-transfected with WT and MT plasmids, the protein variant obstructed the action of wild-type protein in activating the promoter of *OSR2*. However, a similar result was not observed when detecting the luciferase activity of the *StAR* promoter reporter vectors. This result indicated that the dominant negative effect of this novel pathogenic variant would aggravate the dysplasia of eyelid structures. Therefore, considering that the pathogenic variant lost its capacity in binding the promoter of *StAR*, adding its wrong subcellular localization, we speculated that the patient who harbors this pathogenic variant is in a high risk of ovarian insufficiency. However, the occurrence of POI/POF still cannot be predicted

accurately for the reason that no dominant negative effect was observed in the dual-luciferase experiments of *StAR* promoter. Hence, periodical clinical follow-ups and ovarian function assessments are of great importance[24].

In conclusion, we reported a novel *FOXL2* pathogenic variant (c.274G>T) which could be translated into truncated protein (p.E92*). The following *in vitro* biofunctional experiments indicated that the mutant protein cannot distribute correctly, and lost its function as a transcription factor of *StAR* and *OSR2*. The patient with this novel pathogenic variant has a high risk of ovarian insufficiency. The present study not only expands the spectrum of *FOXL2* mutations but also contributes to the understanding of molecular pathogenesis.

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REFERENCES

1 Méjécase C, Nigam C, Moosajee M, Bladen JC. The genetic and clinical features of *FOXL2*-related blepharophimosis, ptosis and epicanthus inversus syndrome. *Genes* 2021;12(3):364.

2 Chawla B, Bhadange Y, Dada RM, Kumar M, Sharma S, Bajaj MS, Pushker N, Chandra M, Ghose S. Clinical, radiologic, and genetic features in blepharophimosis, ptosis, and epicanthus inversus syndrome in the Indian population. *Invest Ophthalmol Vis Sci* 2013;54(4):2985-2991.

3 Rong WN, Ma MJ, Yang W, Yuan SQ, Sheng XL. Identification of a novel FOXL2 mutation in a fourth-generation Chinese family with blepharophimosis-ptosis-epicanthus inversus syndrome. *Int J Ophthalmol* 2021;14(4):504-509.

4 Hu JM, Ke HN, Luo W, Yang YJ, Liu HL, Li GY, Qin YY, Ma JL, Zhao SD. A novel *FOXL2* mutation in two infertile patients with blepharophimosis-ptosis-epicanthus inversus syndrome. *J Assist Reprod Genet* 2020;37(1):223-229.

5 Li F, Chai PW, Fan JY, Wang X, Lu WJ, Li J, Ge SF, Jia RB, Zhang H, Fan XQ. A novel FOXL2 mutation implying blepharophimosis-ptosis-epicanthus inversus syndrome type I. *Cell Physiol Biochem* 2018;45(1):203-211.

6 Castets S, Roucher-Boulez F, Saveanu A, *et al*. Hypopituitarism in patients with blepharophimosis and FOXL2 mutations. *Horm Res Paediatr* 2020;93(1):30-39.

7 Uda M, Ottolenghi C, Crisponi L, *et al*. Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum Mol Genet* 2004;13(11):1171-1181.

8 Tucker EJ. The genetics and biology of FOXL2. *Sex Dev* 2022;16(2-3):184-193.

9 Pisarska MD, Bae J, Klein C, Hsueh AJW. Forkhead l2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. *Endocrinology* 2004;145(7):3424-3433.

10 Kawai S, Michikami I, Kitagaki J, Hashino E, Amano A. Expression pattern of zinc-finger transcription factor Odd-skipped related 2 in murine development and neonatal stage. *Gene Expr Patterns* 2013;13(8):372-376.

11 Bertini V, Valetto A, Baldinotti F, *et al*. Blepharophimosis, ptosis, epicanthus inversus syndrome: new report with a 197-kb deletion upstream of *FOXL2* and review of the literature. *Mol Syndromol* 2019;10(3):147-153.

12 De Baere E, Beysen D, Oley C, *et al*. FOXL2 and BPES: mutational hotspots, phenotypic variability, and revision of the genotype-phenotype correlation. *Am J Hum Genet* 2003;72(2):478-487.

13 Moumné L, Dipietromaria A, Batista F, Kocer A, Fellous M, Pailhoux E, Veitia RA. Differential aggregation and functional impairment induced by polyalanine expansions in FOXL2, a transcription factor involved in cranio-facial and ovarian development. *Hum Mol Genet* 2008;17(7):1010-1019.

14 França MM, Mendonca BB. Genetics of ovarian insufficiency and defects of folliculogenesis. *Best Pract Res Clin Endocrinol Metab* 2022;36(1):101594.

15 Yang Y, Yang C, Zhu Y, *et al*. Intragenic and extragenic disruptions of FOXL2 mapped by whole genome low-coverage sequencing in two BPES families with chromosome reciprocal translocation. *Genomics* 2014;104(3):170-176.

16 Bertho S, Pasquier J, Pan QW, Le Trionnaire G, Bobe J, Postlethwait 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(3):111-129.

17 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 Genom Med* 2018;6(2):261-267.

18 Li F, Chen HF, Wang YF, Yang J, Zhou YX, Song X, Fan JY. Functional studies of novel FOXL2 variants in Chinese families with blepharophimosis-ptosis-epicanthus inversus syndrome. *Front Genet* 2021;12:616112.

19 Beysen D, Moumné 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(13):2030-2038.

20 Tugaeva KV, Sluchanko NN. Steroidogenic acute regulatory protein: structure, functioning, and regulation. *Biochemistry* (*Mosc*) 2019;84(Suppl 1):S233-S253.

21 Kawai S, Abiko Y, Amano A. Odd-skipped related 2 regulates genes related to proliferation and development. *Biochem Biophys Res Commun* 2010;398(2):184-190.

22 Laissue P, Lakhal B, Benayoun BA, *et al*. Functional evidence

implicating FOXL2 in non-syndromic premature ovarian failure and in the regulation of the transcription factor OSR2. *J Med Genet* 2009;46(7):455-457.

23 Kuo FT, Bentsi-Barnes IK, Barlow GM, Pisarska MD. Mutant Forkhead L2 (FOXL2) proteins associated with premature ovarian failure (POF) dimerize with wild-type FOXL2, leading to altered regulation of genes associated with granulosa cell differentiation. *Endocrinology* 2011;152(10):3917-3929.

24 Meng TT, Zhang WZ, Zhang RR, Li J, Gao Y, Qin YY, Jiao X. Ovarian reserve and ART outcomes in blepharophimosis-ptosis-epicanthus inversus syndrome patients with *FOXL2* mutations. *Front Endocrinol* (*Lausanne*) 2022;13:829153.