• Basic Research •

Genetic analysis of Han-Chinese patients with isolated congenital ptosis

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Abstract

● **AIM:** To conduct a genetic analysis of Han-Chinese patients with isolated congenital ptosis (ICP) and identify the genetic variants related to the condition.

● **METHODS:** Sixty-five unrelated patients with ICP were enrolled. Comprehensive clinical examinations, whole exome sequencing (WES), and Sanger sequencing were used to reveal the potential genetic causes. Combined with public and in-house control databases, multiple bioinformatics prediction tools, and conservation analysis, the potential variants were further analyzed. AlphaFold 3, an accurate modelling prediction tool, was utilized to generate three-dimensional structural models of both wild-type and mutated proteins.

● **RESULTS:** Three novel heterozygous variants in the zinc finger homeobox 4 gene (*ZFHX4*), c.5145C>A (p.N1715K), c.10382C>T (p.A3461V), and c.10795G>A (p.A3599T), were identified in three patients, respectively. Bioinformatics analyses suggested that these variants are likely to exert deleterious effects, supporting their potential involvement in the pathogenesis of ptosis.

● **CONCLUSION:** The novel heterozygous *ZFHX4* variants are identified as disease-associated variants in three patients with ptosis, suggesting that *ZFHX4* may be a disease-causing gene for autosomal dominant ICP with incomplete penetrance or a susceptibility gene. These findings expand the variant spectrum of *ZFHX4*, improve understanding of the pathogenesis of *ZFHX4*-related ptosis, and may contribute to the genetic counseling and disease management, as well as the development of experimental treatments.

● **KEYWORDS:** ptosis; *ZFHX4*; missense variants; disease-causing gene; susceptibility gene

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INTRODUCTION

Ptosis is defined as the upper eyelid drooping lower than normal, leading to abnormal palpebral fissure narrowing[1]. It can be classified based on onset time into congenital and acquired ptosis, or by etiology into aponeurotic, myogenic, neurogenic, and mechanical ptosis[2-4]. Congenital ptosis may be detected within the first year of life, which can result in functional, aesthetic, and psychosocial health problems without intervention, as well as compensatory changes[5-6]. It may occur unilaterally or bilaterally and can be either isolated (nonsyndromic) or syndromic, which is often accompanied by other ocular or systemic anomalies such as myasthenia gravis, Marcus Gunn jaw-winking syndrome, and congenital fibrosis of the extraocular muscles[7]. In general, the incidence of ptosis increases with age. The reported rate of childhood ptosis varies

from 0.79 to 1.99 per 10 000 individuals in different regions and populations[7-8]. The prevalence of congenital ptosis ranges from 0.18% to 1.41% in the general population[9-10].

Ptosis patients can be diagnosed through detailed examinations involving clinical neurologic, instrumental, and ophthalmologic investigations, which may guide medical management, rehabilitation, and surgical decision-making[11-12]. Hitherto, the pathogenesis of congenital ptosis and the pattern of gene regulation in eyelid function and pathways remain unclear, which is hypothesized to arise from either a myogenic or neurogenic origin[13]. Nonetheless, genetic testing is more definitive in diagnosing complex or equivocal cases and may potentially increase the chances of targeted prevention and precision treatment, conducive to a better prognosis.

Since the 20th century, studies on monozygotic twins and pedigree analyses have elucidated the involvement of genetic and environmental factors in the pathogenesis of congenital ptosis[14]. To date, only one potential disease-causing gene, the zinc finger homeobox 4 gene (*ZFHX4*, OMIM 606940), and two other loci with unknown causal genes, hereditary congenital ptosis 1 (*PTOS1*, OMIM 178300) and hereditary congenital ptosis 2 (*PTOS2*, OMIM 300245), for isolated congenital ptosis (ICP) have been reported[15-17].

This study enrolled 65 patients with ICP and aimed to identify the disease-associated variant(s) underlying the condition using whole exome sequencing (WES) and Sanger sequencing. Three novel heterozygous missense variants in the *ZFHX4* gene (NM_024721.5), c.5145C>A (p.N1715K), c.10382C>T (p.A3461V), and c.10795G>A (p.A3599T), were identified, in three Han-Chinese patients with ptosis, respectively. Basic Local Alignment Search Tool (BLAST) comparison of protein sequences revealed that the p.N1715, p.A3461, and p.A3599 are highly conserved in the ZFHX4 protein, and structural modelling also indicates that these residues are crucial to the structure and function of the ZFHX4 protein. These findings may expand the variant spectrum of *ZFHX4* and enhance understanding of pathogenesis, accurate diagnosis, and novel therapeutics.

PARTICIPANTS AND METHODS

Ethical Approval The study was approved by the Institutional Review Board of the Third Xiangya Hospital (No.2018-S020), with informed consent obtained from participants or guardians, strictly following the guidelines of the Declaration of Helsinki.

Participants This study enrolled 65 unrelated Han-Chinese patients with ptosis and included 650 controls from our in-house database. Medical records of affected individuals for historical complaints related to ptosis were reviewed, and comprehensive assessments and interviews were conducted by one or more clinicians. No related signs of ptosis and negative related family history were confirmed in the controls.

WES and Bioinformatics Analysis Genomic DNA (gDNA) was isolated from the sampled peripheral venous blood of the 65 patients using phenol-chloroform extraction protocols, and WES was performed for pathogenic variants[18]. The gDNA was randomly fragmented by ultrasonic technique as required. These fragments were end-repaired, subjected to dA-tailed reactions, and further ligated with adapters. The prepared samples were amplified *via* ligation-mediated polymerase chain reaction, purified and hybridized to an exome array for enrichment, and circularized in accordance with protocols. DNA nanoballs were generated by rolling circle amplification, and qualified captured library was loaded onto BGISEQ-500 or DNBSEQ platforms (BGI-Shenzhen, Shenzhen, China) for high-throughput sequencing[19-20]. After the raw data in FASTQ format were filtered to generate clean data, the alignment to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner was conducted, and potential duplicate paired-end reads were removed using Picard or Genome Analysis Toolkit MarkDuplicates tool. Sequencing depth and coverage were assessed per individual, with stringent quality control system measures executed. The Genome Analysis Toolkit BaseRecalibrator, ApplyBQSR, and HaplotypeCaller tools were used for base quality score recalibration and accurate variant calling. High-quality and confident variants were obtained using the hard-filtering procedure, followed by further annotation using the SnpEff tool[21]. Candidate variants were filtered using the following databases: Single Nucleotide Polymorphism database (build 157, dbSNP157), 1000 Genomes Project (1000G), Exome Sequencing Project 6500 (ESP6500), Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD), China Metabolic Analytics Project (ChinaMAP), ClinVar, and Human Gene Mutation Database (HGMD), and the BGI in-house exome database, as well as our in-house exome database with 650 Chinese controls[20].

Sanger Sequencing Sanger sequencing was performed to validate the potential pathogenic variants identified by WES with an ABI 3730XL sequencer (Applied Biosystems Inc., Carlsbad, CA, USA)[22]. Primer3 (https://primer3.ut.ee) was used to design custom polymerase chain reaction amplification primers and sequencing primers for detecting the candidate variants (Table 1), and primer specificity was verified using primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)[23].

Variant Evaluation, Conservation Analysis, and Structure Modelling Bioinformatics prediction programs, including Combined Annotation Dependent Depletion, MutationTaster, Sorting Intolerant from Tolerant, Polymorphism Phenotyping v2, and Protein Variation Effect Analyzer, were used to estimate the pathogenicity of the variants[24-28]. The protein

Table 1 Primers used for identification of the *ZFHX4* gene variants

Variant	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Product size
c.5145C>A	ACCACAGTCACCAGCACAAA	CTGAACTCCGTCCCAGGTAT	186 bp
c.10382C>T	TGACCCACAAGAGACAGTGC	TGCAGACTGCGAGGTAGATG	226 bp
c.10795G>A	TCAAGTTTGTGCAGCACCTC	CACGGGATCCTGTCTTCACT	207 bp

ZFHX4: The zinc finger homeobox 4 gene.

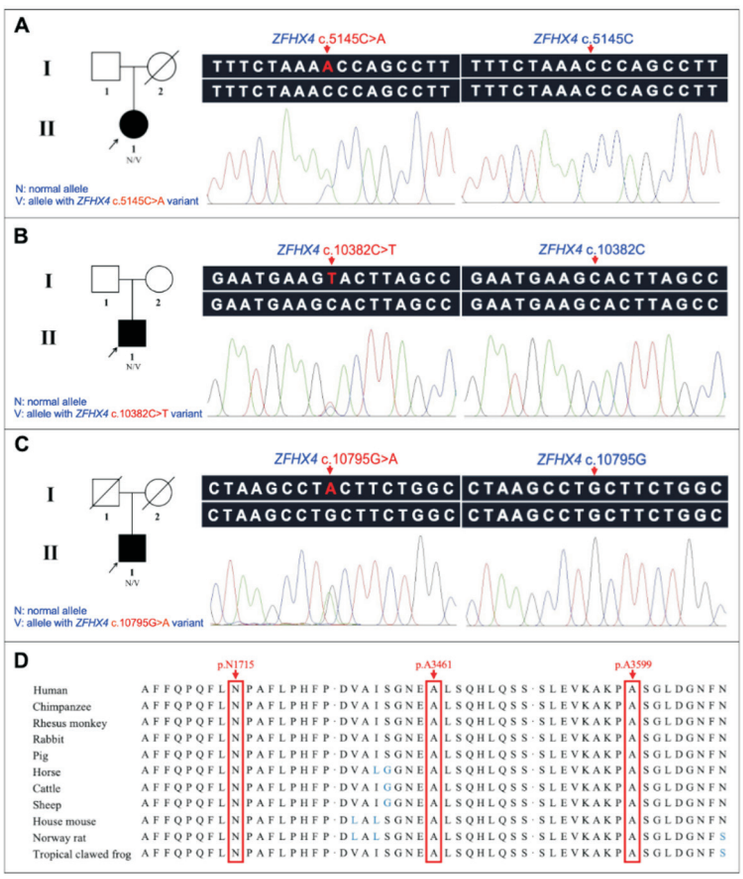

Figure 1 Pedigrees and sequence analysis of the three unrelated Han-Chinese patients with ptosis A-C: Pedigrees with ptosis and Sanger sequencing results of the probands with a *ZFHX4* heterozygous variant and controls with a normal *ZFHX4* sequence. In the pedigrees, black symbols indicate affected individuals, white symbols indicate unaffected individuals, slanted line symbols indicate deceased individuals, and arrows point to probands. D: Protein sequence alignment of the zinc finger homeobox 4 among different species, with the affected amino acids indicated by the arrows. *ZFHX4*: The zinc finger homeobox 4 gene.

BLAST was utilized to analyze the sequences in 11 species[29]. Predictions and visualizations of wild-type and variant-type protein structures were achieved using the AlphaFold 3 and Visual Molecular Dynamics software, respectively[30-31].

RESULTS

Clinical Findings Among the 65 patients, 31 were male and 34 were female. A total of 29 patients (44.62%) had unilateral ptosis (left/right: 19/10), and 36 (55.38%) had bilateral ptosis. All enrolled patients presented with abnormal upper eyelid drooping and a narrowed vertical palpebral fissure. Additionally, a few patients (4.62%) exhibited compensatory changes, including backward-tilted head, forehead swelling, and elevated eyebrows.

Genetic Findings The gDNA samples of 65 patients were subjected to WES, and three potential pathogenic variants in the *ZFHX4* gene were identified in three patients (Figure 1A-1C), respectively. Patient 1 (II:1, Figure 1A) was a 52-year-old female showing bilateral ptosis, with margin-reflex distance 1 (MRD1) 1-2 mm and fair levator function (5 mm). Patient 2 (II:1, Figure 1B) was a 50-year-old male who had bilateral ptosis with blurred vision, limited extraocular motility, MRD1<1 mm, and poor levator function (2-3 mm). Patient 3 (II:1, Figure 1C) was a 58-year-old male presenting unilateral ptosis, with MRD1<1 mm and poor levator function (2 mm) in his left eyelid.

WES of the three patients generated an average of 100.51 million (121.96 million, 86.14 million, and 93.43 million, respectively) clean reads, and over 99% (99.93%, 99.99%, and 99.98%, respectively) of the reads were mapped to the human reference genome. The average sequencing depth of the target

Table 2 Identification of the *ZFHX4* gene variants in the three patients

Category	Variant 1	Variant 2	Variant 3
Patient	Proband 1	Proband 2	Proband 3
Exon	10	11	11
Nucleotide change	c.5145C>A	c.10382C>T	c.10795G>A
Amino acid change	p.N1715K	p.A3461V	p.A3599T
Zygosity	Heterozygous	Heterozygous	Heterozygous
Variant type	Missense	Missense	Missense
dbSNP157	No	No	rs1485304986
1000G	No	No	No
ESP6500	No	No	No
ExAC	No	No	No
gnomAD	No	No	No
ChinaMAP	No	No	No
In-house exome database	No	No	No
ClinVar	No	No	No
HGMD	No	No	No
CADD v1.7 (phred score)	Deleterious (23.2)	Deleterious (28.6)	Deleterious (23.4)
MutationTaster	Disease causing	Disease causing	Disease causing
SIFT (score)	Damaging (0.002)	Damaging (0.002)	Damaging (0.004)
PolyPhen-2 (score)	Possibly damaging (0.766)	Probably damaging (0.987)	Probably damaging (0.954)
PROVEAN (score)	Deleterious (-4.19)	Deleterious (-2.62)	Neutral (-1.99)

ZFHX4: The zinc finger homeobox 4 gene; dbSNP157: Single Nucleotide Polymorphism database build 157; 1000G: 1000 Genomes Project; ESP6500: Exome Sequencing Project 6500; ExAC: Exome Aggregation Consortium; gnomAD: Genome Aggregation Database; ChinaMAP: China Metabolic Analytics Project; HGMD: Human Gene Mutation Database; CADD: Combined Annotation Dependent Depletion; SIFT: Sorting Intolerant from Tolerant; PolyPhen-2: Polymorphism Phenotyping v2; PROVEAN: Protein Variation Effect Analyzer.

region was 153.17-fold (Patient 1), 114.57-fold (Patient 2), and 107.49-fold (Patient 3). Of these sequences, 98.51%, 98.32%, and 98.35% of the target regions were covered by at least 20×, respectively. For the annotated variants, 125 196 single nucleotide polymorphisms (SNPs) and 19 204 insertions-deletions (indels) in Patient 1, 130 305 SNPs and 25 246 indels in Patient 2, and 134 198 SNPs and 26 406 indels in Patient 3, were detected.

Variant Analysis These variants were absent from several public databases, 1000G, ESP6500, ExAC, gnomAD, ChinaMAP, ClinVar, and HGMD, as well as in-house exome databases. Three novel heterozygous variants, c.5145C>A (p.N1715K), c.10382C>T (p.A3461V), and c.10795G>A (p.A3599T), in the known ICP gene, *ZFHX4*, were considered as potential pathogenic variants in the three patients, respectively. Sanger sequencing revealed the presence in the patients and the absence in in-house controls (Figure 1A-1C). Bioinformatics prediction programs further revealed that these variants have deleterious effects (Table 2). BLAST comparison of protein sequences from tropical clawed frog to human indicated that the amino acid residues at the mutated positions, p.N1715, p.A3461, and p.A3599, are conserved (Figure 1D). Three-dimensional structural models showed the wild-type and variant-related conformations (Figure 2). These combined findings further support that the variants are pathogenic, with no other likely ptosis-associated variants in the three patients noted.

DISCUSSION

ICP refers to the abnormal drooping of the upper eyelid, which causes a diminution in the vertical extent of the palpebral fissure and has the potential to impair visual development and function, as well as other health issues[17,32]. The condition can manifest as sporadic or familial, with sporadic cases accounting for the majority and small families being not uncommon[5,10,15]. The etiology is likely multifactorial, involving genetic and environmental factors that contribute to developmental myogenic and/or neurogenic defects. Variants in a single gene or multiple genes, like acting as a disease-causing, susceptibility, or modifier gene, may be implicated[13,33]. In a five-generation autosomal dominant ICP family with 14 patients, the variable ptosis severity and laterality (unilateral/bilateral ptosis: 11/3, left unilateral preponderance: 9/11) suggested a possible involvement of variants in a single disease-causing gene and a modifier gene[34]. Additionally, autosomal dominant transmission in a four-generation ptosis family supported the genetic contribution, while the discordant phenotypes in monozygotic twins further supported that uncertain prenatal environmental exposures or epigenetics

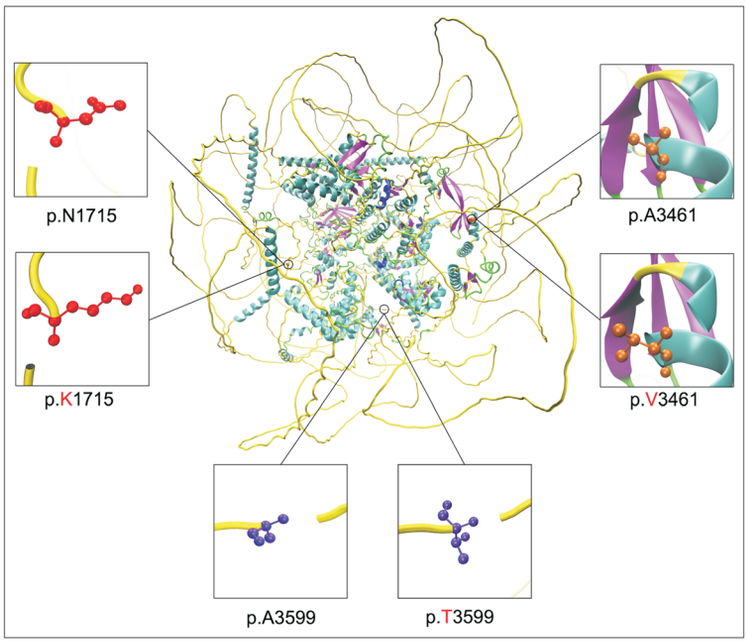

Figure 2 Cartoon model of the zinc finger homeobox 4 protein structure, with the residues at the mutated positions further shown as ball-and-stick models.

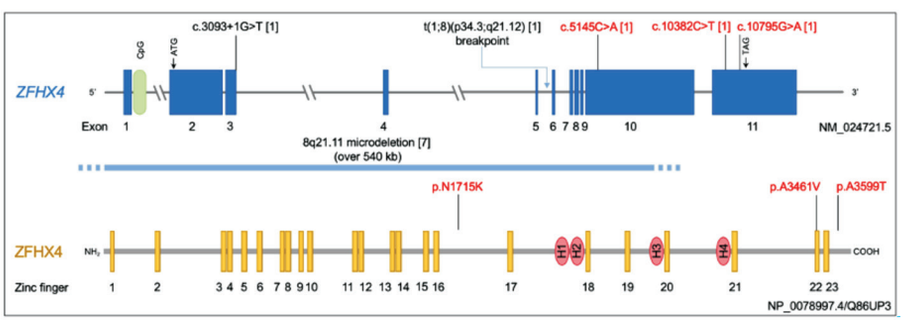

Figure 3 Schematic distribution of variants involving *ZFHX4* associated with ptosis The upper shows the 11 exons in the *ZFHX4* gene, with numbers in square brackets following the variants representing the reported number of patients. The bottom shows 23 zinc finger domains and 4 homeodomains (H1-H4) of full-length ZFHX4 (3616 amino acids). Variants identified in this study are highlighted in red. *ZFHX4*: The zinc finger homeobox 4 gene.

may also exert a critical effect[35]. Hitherto, only abnormalities involving the *ZFHX4* gene have been reported in association with congenital ptosis (Figure 3). In 2002, a *de novo* balanced chromosomal translocation, 46,XY,t(1;8)(p34.3;q21.12), disrupting the *ZFHX4* gene, was reported in a 7-month-old boy with bilateral ICP[17]. The 8q21.11 microdeletion, involving the functional gene *ZFHX4*, was found in eight unrelated individuals with a syndrome characterized by intellectual disability and recognized phenotypes like ptosis (7/8 cases)[36]. Additionally, a *de novo* heterozygous *ZFHX4* c.3093+1G>T variant was identified in a 10-year-old girl with neuropsychological and facial phenotypes, including ptosis[37]. The disease-causing gene *ZFHX4*, mapped to chromosome 8q21.13, spans over 186 kb and comprises 11 exons, with a large coding exon 10. The gene encodes an about 399-kDa protein with 3616 amino acids, which is broadly expressed in various tissues such as brain and muscle, with a known function in neural and muscle differentiation[38]. There are three other ZFHX proteins in vertebrates, ZFHX1, ZFHX2, and ZFHX3, which are transcription factors with characteristic zinc finger domains and homeodomains[39]. Human ZFHX4 is a potential DNA-binding protein with 23 C2H2 zinc fingers, each with a corresponding sequence of 20-25 amino acids, related to 6 exons, and 4 homeodomains encoded by exon 10 (Figure 3). The fundamental C2H2 zinc finger, the most classic type, chelates a zinc ion with conserved cysteine and histidine residues in a stabilized beta-beta-alpha structure, with a DNA-, RNA-, or protein-binding capacity. The capacity is determined by the sequences of zinc finger domains and inter-domain linkers, and the higher-order structures and the number of zinc fingers[40]. The typical homeodomain, a DNA-binding domain, is composed of about 60 amino acids, forms a globular structure with three alpha helices, and functions in recognizing the specific DNA sequences and regulating the

downstream gene transcription. *ZFHX4* expression is regulated by transcription factors including sex-determining region Y protein, caudal-type homeodomain protein, and myeloid zinc finger protein 1, with the similar regulation observed in mouse *Zfhx4*. Rat Zfhx4 protein is expressed in the oculomotor nucleus of adult brain[38], which is related to the complex oculomotor system and ptosis-related anatomy, including the oculomotor nerve and its innervated levator palpebrae superioris[41]. Additionally, the ZFHX4 protein acts as a molecular regulator and can bind to chromodomain helicase DNA binding protein 4 (CHD4), a core component of the nucleosome remodeling and deacetylase complex, involved in stem cell maintenance and cancer progression[42]. Of interest, the mouse paired like homeobox 2B gene (*Phox2b*) is a known direct downstream target of *Zfhx4*, and the human *PHOX2B* paralog, the paired like homeobox 2A gene (*PHOX2A*), is a disease-causing gene of congenital fibrosis of the extraocular muscles 2, which includes ptosis as a phenotype. The encoded proteins, PHOX2A and PHOX2B, are transcription factors and have identical 63-amino-acid homeodomains[39]. The potential relationship between the only isolated ptosis-associated gene (*ZFHX4*) and its downstream target gene (*PHOX2B*) was analyzed using GeneMANIA[43], and the potentially related genes included the *ZFHX4* paralogs (*ZFHX2* and *ZFHX3*), the ZFHX4's regulatory target protein coding gene *CHD4*, and the *PHOX2B* paralog *PHOX2A*. Therefore, it is reasonable to speculate that these genes may be associated with ptosis or involve in syndromes featuring ptosis.

In this study, three novel heterozygous variants in the *ZFHX4* gene, c.5145C>A (p.N1715K), c.10382C>T (p.A3461V), and c.10795G>A (p.A3599T), absent in controls and predicted to be harmful, were considered to be responsible for ptosis in three Han-Chinese patients. All three variants are consistent with the variant types reducing hydrogen bonds, from three bonds in C-G to two bonds in T-A, by lowering energy for DNA double-strand separation and mRNA stem-loop disruption in DNA replication, transcription, and translation processes[44]. They all lead to larger side-chain residue substitutions, which may increase steric hindrance among surrounding residues, changing the charge state, hydrophobicity, secondary structure, or related accessibility, and affect the conformational stability, as well as the function and molecular interaction, *via* the direct or indirect impact on zinc fingers. The p.N1715K variant is located in an important inter-domain linker between zinc finger domains 16 and 17 and involves a substitution from uncharged asparagine to positively charged lysine with a relatively larger side chain, leading to the loss of helices and the alteration of relative accessibility between "accessible" and "intermediate" in regions encompassing the residue (analyzed by FoldScript, https://foldscript.ibcp.fr)[45], hypothesized to induce a decline in conformational stability. The p.A3461V variant is located within the critical zinc finger domain 22 and leads to the valine with a relatively larger side chain, inducing the transition of relative accessibility from "intermediate" to "accessible", as well as the alteration in surroundings[45], which may affect local conformation and molecular interactions, disrupt protein structural stability, and interfere with functions such as transcriptional regulation. The p.A3599T variant is located at the C-terminus and involves a substitution of hydrophobic alanine to hydrophilic threonine with a relatively larger side chain, causing the alteration of relative accessibility between "accessible" and "intermediate" in regions surrounding the residue[45], probably affecting protein structural stability.

The relatively large molecular mass of ZFHX4 may hinder in-depth study on its function and the exact *ZFHX4*-related mechanism of ptosis remains seriously lacking. Heterozygous *Zfhx4*$^{+/-}$ mice showed no obvious abnormalities, while homologous *Zfhx4*$^{-/-}$ mice died within 24h. Studies on *Zfhx4*-deficient mouse models are limited and ptosis is easily overlooked[39]. Constructing site-specific gene-deficient animal models and conducting further functional studies will help to facilitate phenotypic mimicking and development of experimental treatments based on genetic findings.

In summary, three novel heterozygous *ZFHX4* variants, c.5145C>A (p.N1715K), c.10382C>T (p.A3461V), and c.10795G>A (p.A3599T), were identified in Han-Chinese patients with ICP *via* WES and Sanger sequencing. Combined with bioinformatics analysis findings, a suggestive association of *ZFHX4* missense variants with ptosis was found, indicating that the *ZFHX4* gene may be an autosomal dominant disease-causing gene for ptosis with incomplete penetrance or act as a susceptibility gene. Our study expands the variant spectrum of *ZFHX4* and may contribute to improved genetic counseling, prenatal diagnosis, and disease management for the families. Owing to the small size of the families and the large molecular mass of the protein, familial segregation analysis and corresponding functional study are impracticable. Additionally, the study is limited by the availability of current resources, including the scanty knowledge of the *ZFHX4* gene and its encoding protein and the reported small number of potential pathogenic variant carriers, as well as the vague genetic cause and pathogenesis of ICP. Future research involving large-scale screenings and multi-center studies to identify more *ZFHX4* variants in ptosis cases (especially isolated individuals) may help unravel the genetic basis and molecular mechanism of this enigmatic disorder. Developing site-specific gene-deficient animal models and conducting gene-associated functional studies will facilitate a deeper understanding of the cellular and molecular mechanisms associated with *ZFHX4* and ptosis, and develop experimental treatments.

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Authors' Contributions: Conceived and designed this study: Zhang QL, Yuan LM, and Deng H; Collected the patient samples and clinical data: Zhang QL, Yuan LM, Zheng W, Yi JH, and Deng H; Performed the experiments and analyzed the data: Zhang QL, Yuan LM, Deng XY, Yi JH, Xu HB, and Deng H; Wrote the manuscript: Zhang QL, Yuan LM, Deng XY, and Deng H. The final version of the manuscript was read and approved by all authors.

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Conflicts of Interest: Zhang QL, None; **Yuan LM,** None; **Deng XY,** None; **Zheng W,** None; **Yi JH,** None; **Xu HB,** None; **Deng H,** None.

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