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Identification of truncated variants in *GLI* family zinc finger 3 (*GLI3*) associated with polydactyly

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Abstract

Background Polydactyly is a prevalent congenital anomaly with an incidence of 2.14 per 1000 live births in China. *GLI* family zinc finger 3 (*GLI3*) is a classical causative gene of polydactyly, and serves as a pivotal transcription factor in the hedgehog signaling pathway, regulating the development of the anterior-posterior axis in limbs.

Methods Three pedigrees of polydactyly patients were enrolled from Hunan Province, China. Pathogenic variants were identified by whole-exome sequencing (WES) and Sanger sequencing.

Results Three variants in *GLI3* were identified in three unrelated families, including a novel deletion variant (c.1372del, p.Thr458GlnfsTer44), a novel insertion-deletion (indel) variant (c.1967_1968delinsAA, p.Ser656Ter), and a nonsense variant (c.2374 C > T, p.Arg792Ter). These variants were present exclusively in patients but not in healthy individuals.

Conclusions We identified three pathogenic *GLI3* variants in polydactyly patients, broadening the genetic spectrum of *GLI3* and contributing significantly to genetic counseling and diagnosis for polydactyly.

Keywords Polydactyly, *GLI3*, Truncated variant, Isolated polydactyly, Greig cephalopolysyndactyly syndrome

Background

Polydactyly is a common congenital limb anomaly, occurring in approximately 2.14 per 1000 live births in China [1]. Polydactyly includes syndromic polydactyly and isolated polydactyly (IPD). IPD is further classified into three categories: preaxial polydactyly, which involves a non-functional duplicated thumb on the hand or foot; postaxial polydactyly, featuring a fully or partially duplicated digit on the ulnar side of the hand or foot; and central polydactyly, a less common type involving anomalies of the index, middle, or ring fingers [2]. Surgical interventions, such as removal of supernumerary digits and redundant skeletal structures, are the primary treatments to maintain joint stability and soft tissue balance [3]. Various genes have been identified as contributing to IPD, including the *GLI* family zinc finger 3 (*GLI3*, OMIM 165,240), *zinc finger protein 141* (*ZNF141*,

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OMIM 194,648), *IQ domain-containing protein E (IQCE*, OMIM 617,631), *GLI family zinc finger 1 (GLI1*, OMIM 165,220), *Family with sequence similarity 92, member A (FAM92A*, OMIM 617,273), *KIAA0825* (OMIM 617,266), *Dachshund family transcription factor 1 (DACH1*, OMIM 603,803), *Mirror-image polydactyly 1 (MIPOL1*, OMIM 606,850), and *Paired-like homeodomain 1 (PITX1*, OMIM 602,149) [4–12]. Notably, *GLI1* and *GLI3* are crucial for limb development, with *GLI3* being a classical gene implicated in polydactyly [13].

The *GLI3* gene, located on chromosome 7p14.1, encodes an 1580-amino acid protein that exists in two isoforms: the full-length activator (GLI3-A) and the truncated repressor (GLI3-R) [14]. The GLI3 protein plays a pivotal role in limb development by acting as a nuclear transducer and negative regulator of Sonic Hedgehog (SHH) signaling, which establishes the zone of polarizing activity essential for the formation of the anterior-posterior limb axis [15, 16]. Loss-of-function variants in *GLI3* can lead to various limb development disorders, including Greig cephalopolysyndactyly syndrome (GCPS; OMIM 175,700), preaxial polydactyly type A/B (PAPA/PAPB; OMIM 174,200), Pallister Hall syndrome (PHS; OMIM 146,510), postaxial polydactyly type A1 and B (OMIM 174,200) and preaxial polydactyly type III (OMIM 174,700) [17–20].

In this study, we recruited several families with polydactyly and identified three *GLI3* variants, including a novel deletion variant (NM_000168.6: c.1372del, p.Thr458GlnfsTer44), a novel insertion-deletion (indel) variant (NM_000168.6: c.1967_1968delinsAA, p.Ser656Ter), and a previously reported nonsense variant (NM_000168.6: c.2374 C>T, p.Arg792Ter). These findings broaden the genetic spectrum associated with *GLI3* and enhance genetic counseling and diagnosis of polydactyly.

Methods

Ethical compliance

This study was approved (2,023,030,444) by the Ethics Committee of Xiangya Hospital, Central South University, Changsha, China. We performed this study in accordance with the principles outlined in the Declaration of Helsinki. The patients/participants or their guardians provided written informed consent to participate in the study.

Participants/patients

Three families (Family I-III) were investigated in this study. Peripheral blood samples were collected from probands and their family members. Clinical data were recorded carefully.

Whole-exome sequencing

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Exome capture and whole-exome sequencing (WES) were conducted at Berry Genomics (Beijing, China). 1 µg DNA was captured using the SureSelect Human All Exon Kit V6 (Agilent Technologies, Inc., CA, USA) and sequenced using the Illumina HiSeq4000 platform (Illumina Inc., CA, USA). Briefly, the genomic DNA was randomly extracted using a Covaris S220 sonicator (Covaris, Inc., MA, USA). The fragmented DNA underwent three enzymatic steps: end repair, a-tailing, and adapter ligation. The adapter-ligated DNA fragments were amplified using Hercules II Fusion DNA Polymerase (Agilent). Finally, the exomes in the pre-capture libraries were captured using the SureSelect capture library kit (Agilent). After DNA quality assessment, the captured DNA library was subjected to WES on the Illumina HiSeq4000 platform. Downstream processing was carried out using the Genome Analysis Toolkit (GATK), Varscan2, and Picard, and variant calls were made with the GATK Haplotype Caller 12. Variant annotation was performed according to Ensemble release 82, and filtering was conducted using ANNOVAR Documentation.

The filtering strategies conformed to those used in our previous study [21]. Variants with an alternative allele frequency > 0.001 in the 1000G database (<http://www.1000genomes.org/>) and the GnomAD database (<https://gnomad.broadinstitute.org/>) were used for further analysis. These filtered variants were predicted their pathogenicity using MutationTaster (<http://www.mutationtaster.org/>), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://provean.jcvi.org/index.php>), and CADD (<https://cadd.gs.washington.edu/snv>) [22]. Bone development related genes were used to filter candidate variants [23].

Co-segregation analysis

Co-segregation analysis was performed on each family member using Sanger sequencing. The primer pairs (GLI3-1-F: 5'-CCTCCTGTTGTGTCTGATTCTT-3'; GLI3-1-R: 5'-GGTTCCTGAATACCATCCACTT-3'; GLI3-2-F: 5'-GAGGCTCATGTCACCAAGAA-3'; GLI3-2-R: 5'-CTGTGAAGTCAGAAGGAGAGTG-3'; GLI3-3-F: 5'-CCAAATGGATGGAGCACGTA-3'; GLI3-3-R: 5'-CGGATGGTTACAGCGTCATT-3') used for PCR amplification were designed using Primer 5. The sequences of the PCR products were determined using an ABI 3100 Genetic Analyzer (ABI, Foster City, CA, USA) [22].

Table 1 Clinical details of patients in this study

		Family I				Family II	Family II
		Proband I	Mother	Grandfather	Sister	Proband II	Proband III
Upper limb	Polydactyly	-	-	-	-	Bil	-
	Syndactyly	Bil (4-5)	-	-	Bil	-	-
Lower limb	Polydactyly	Bil (1)	Bil	Bil	-	Bil	Bil (1,5)
	Syndactyly	-	-	-	Bil	-	Bil (1-2)

Bil, bilateral; 1, thumb or toe; 2/4/5, the second/fourth/fifth finger

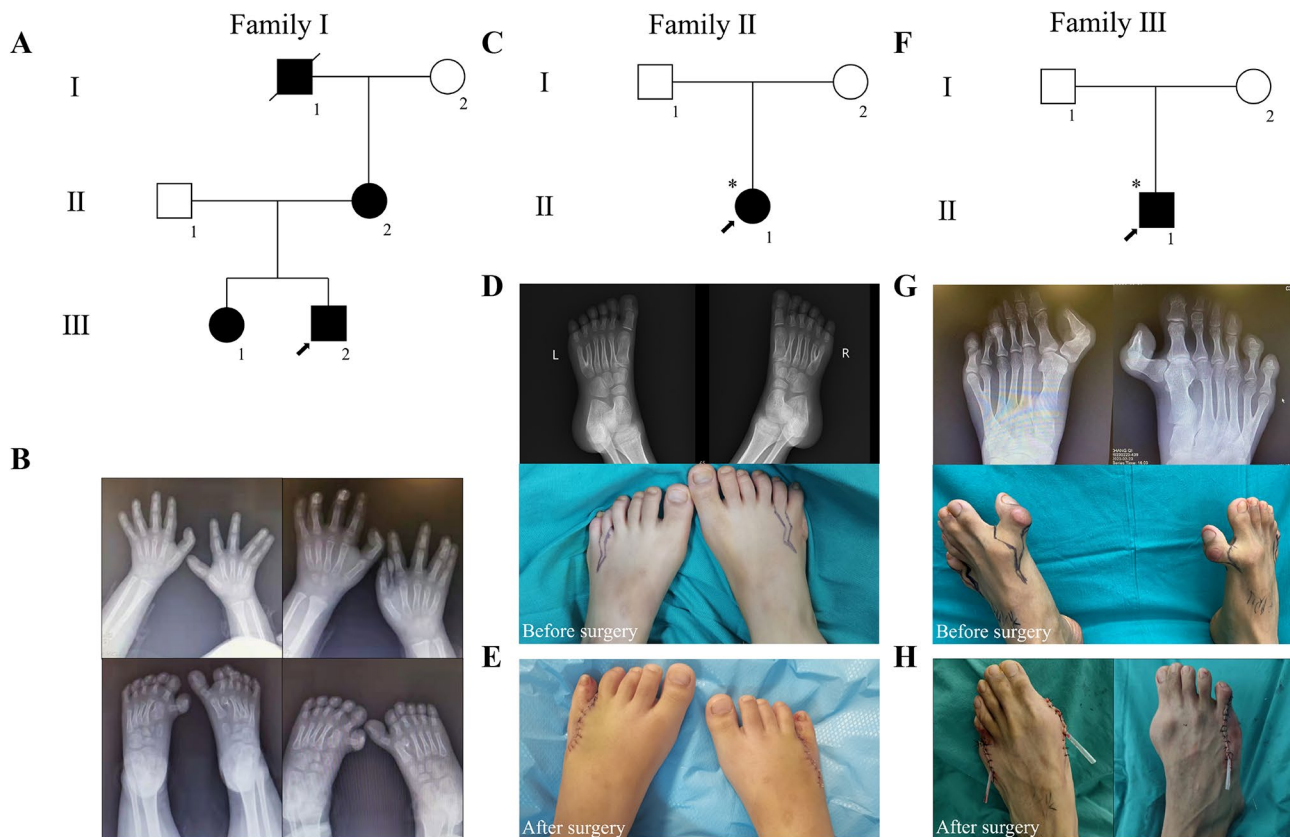


Fig. 1 Clinic description of the probands with polydactyly related to GLI3. **(A)** Pedigree of Family I. **(B)** Clinical features of proband I. **(C)** Pedigree of Family II. **(D)** Clinical features of proband II before surgery. **(E)** Clinical features of proband II after surgery. **(F)** Pedigree of Family III. **(G)** Clinical features of proband III before surgery. **(H)** Clinical features of proband III after surgery. Squares = men; circles = women; black symbols = individuals with variants; Slashes = individuals who die; arrows = the probands

Results

Clinical description

We conducted an investigation into three Chinese families (Family I-III) from Hunan Province of China (Table 1). Family I consists of six immediate members, four of whom have exhibited features of polydactyly or syndactyly (Fig. 1A). Radiography showed that the proband presented syndactyly of the fourth and fifth fingers on the right hand and polydactyly of thumbs on each foot (Fig. 1B). Additionally, his sister was affected by syndactyly on all limbs, and his mother had surgically corrected dysmorphic toes. The family reported a history of polydactyly in the deceased grandfather's toes.

Proband II from Family II was born with polydactyly affecting both hands and feet, with no similar conditions reported in the family's history (Fig. 1C and D). Surgical removal of the extra fingers was performed at a different hospital when the patient was one year old, and subsequent toe resection occurred at our hospital, resulting in successful recover (Fig. 1E).

Proband III from Family III displayed heptadactyly on both feet, polydactyly on the first and fifth toes, and syndactyly between the first and second toes, with no reported familial history of these anomalies (Fig. 1F and G). The extra toes were surgically removed (Fig. 1H). Considering the clinical manifestations in Family I and

III, we made primary diagnosis of mild GCPS, whereas the proband from Family II appeared to have IPD [13].

Genetic analysis

Using WES and Sanger sequencing, we identified three *GLI3* variants in the probands from three families, including two novel variants (NM_000168.6: c.1372del, p.Thr458GlnfsTer44 and c.1967_1968delinsAA, p.Ser656Ter) and one known variant (NM_000168.6: c.2374 C>T, p.Arg792Ter). According to the standards of the American College of Medical Genetics and Genomics (ACMG) standards, these variants were designated as “pathogenic” (Table 2).

In Family I, WES produced 9.7 Gb of data, achieving 98.1% coverage of the target region and over 10× coverage for 99.0% of the targets. After excluding common variants in the 1000G and GnomAD databases, and retaining variants predicted to be disease-causing using MutationTaster, Polyphen-2, SIFT and CADD, which were also positioned in genes associated with bone development, one *GLI3* variant (c.1372del, p.Thr458GlnfsTer44) was identified. Sanger sequencing further confirmed the heterozygous frameshift variant in *GLI3* in Proband I (Fig. 2A). Adherence to ACMG guidelines, this variant was deemed “pathogenic”: it was a frameshift variant often leading to loss of function (PVS1), was absent in control cohorts from the 1000G and GnomAD databases (PM2) and co-segregated with the polydactyly phenotype in Family I (PP1). It was also predicted to have a deleterious effect on the gene product as determined by tools such as MutationTaster (PP3). Additionally, alignments of *GLI3* amino acid sequences across various species showed high conservation in this region (p.458–466) (Fig. 2B), demonstrating the critical nature of this sequence.

In Family II, we identified the heterozygous nonsense variant (c.1967_1968delinsAA, p.Ser656Ter) in Proband

II (Fig. 2C). According to the ACMG guidelines, this variant was classified as a nonsense variant (PVS1). It has not been observed in healthy population databases (PM2), and bioinformatic tools predict its deleterious effects (PP3). Consequently, it was designated as “pathogenic”.

In Family III, a heterozygous variant (c.2374 C>T, p.Arg792Ter) was identified, which was a known truncation variant (Fig. 2D) [24]. Similarly, according to the ACMG guidelines (PVS1, PM2, PP3), this variant was considered “pathogenic”.

Discussion

GLI3 is a pivotal transcription factor in the SHH signaling pathway, comprising multiple domains: a repressor domain (RD), a suppressor of fusion (SUFU) site, a zinc finger domain (ZFN), a cleavage site (CS), a CREB-binding protein (CBP) domain, and two transactivation domains (TAD1 and TAD2). The ZFN domain facilitates *GLI3*'s DNA binding, enabling transcriptional suppression or activation of SHH target genes. Additionally, the CS domain allows *GLI3* to be cleaved from *GLI3*-A to *GLI3*-R by the proteasome [25–28]. In this study, we identified three *GLI3* variants (c.1372del, p.Thr458GlnfsTer44, c.1967_1968delinsAA, p.Ser656Ter, and c.2374 C>T, p.Arg792Ter) in polydactyl patients. The variant p.Thr458GlnfsTer44, located in the ZFN domain, disrupts transcriptional functions, while variants p.Ser656Ter and p.Arg792Ter, positioned in or adjoining the CS, directly influence the *GLI3*-A to *GLI3*-R conversion (Fig. 2E).

It has been suggested that the location of *GLI3* variants significantly impacts the manifestation of digital anomalies due to the dual role of *GLI3* as an activator or repressor in the SHH pathway [29]. In mouse model, *GLI3* haploinsufficiency, often resulting from loss-of-function variants upstream of or within the

Table 2 Information and pathogenicity classification of *GLI3* variants in this study

Patient	Gene	Variant	Pathogenicity prediction	GnomAD	1000G	OMIM clinical phenotype	American College of Medical Genetics classification
Proband I	<i>GLI3</i>	NM_000168.6: c.1372del, p.Thr458GlnfsTer44	MutationTaster: D Polyphen-2:- SIFT: - CADD: -	-	-	AD; Polydactyly, postaxial, types A1 and B; Polydactyly, preaxial, type IV	Pathogenic (PVS1, PM2, PP1, PP3)
Proband II	<i>GLI3</i>	NM_000168.6: c.1967_1968delinsAA, p.Ser656Ter	MutationTaster: D Polyphen-2: - SIFT: - CADD: -	-	-		Pathogenic (PVS1, PM2, PP3)
Proband III	<i>GLI3</i>	NM_000168.6: c.2374 C>T, p.Arg792Ter	MutationTaster: D Polyphen-2:- SIFT: - CADD: 37	-	-		Pathogenic (PVS1, PM2, PP3)

D, disease causing; AD, autosomal recessive

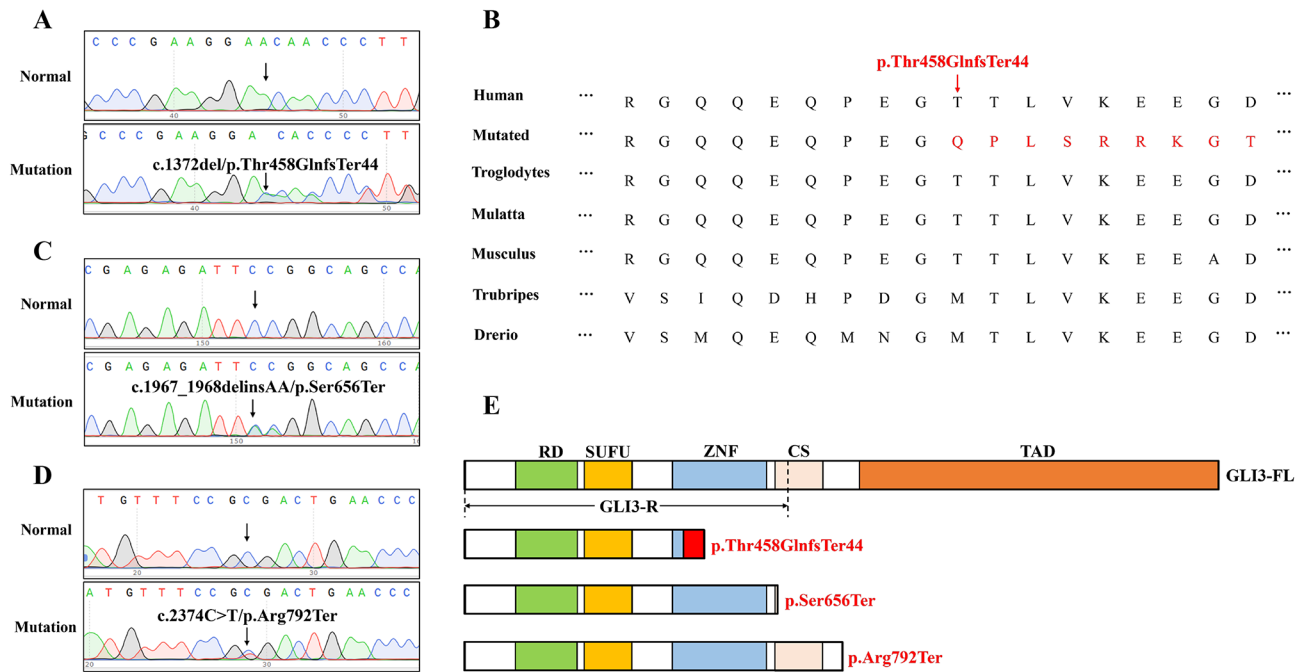


Fig. 2 Genetic description of the probands with polydactyly related to GLI3. **(A, C, D)** Sanger sequencing results of the GLI3 variant among the probands. **(B)** Alignment analysis of the region (p.458–466) in the GLI3 amino acid sequence showed that the region was highly conserved. **(E)** Localization of the variant in GLI3. Red region = frameshift variant; RD = repressor domain; SUFU = SUFU site; ZFN = zinc finger domain; CS = cleavage site, TAD = transactivation domain

ZFN domain, has been linked to the pathogenesis of GCPS, characterized by cranial enlargement and a wide interorbital distance [30]. In this study, the frameshift variant p.Thr458GlnfsTer44 in Family I introduces a premature stop codon, leading to a GLI3 protein lacking the essential ZFN domain for DNA binding, which may contribute to GCPS, corroborating earlier research findings. The CS domain is critical in regulating the ratio of GLI3-A/GLI3-R, thus, variants in the CS region can result in an excess of GLI3-R in limb buds and the neural tube, which predominantly drives the pathogenesis of PHS. PHS is associated with diverse anomalies including hypothalamic hamartoma, cleft larynx, imperforate anus, and pulmonary lobation anomalies [31]. This study also identified two truncation variants (p.Ser656Ter and p.Arg792Ter) within the CS domain, which may result in premature termination of GLI3, loss of GLI3-A function, and excessive production of GLI3-R (Fig. 2E). Notably, while the patient in Family II was diagnosed with IPD, and the patient in Family III with mild GCPS, neither presented craniofacial anomalies. Research by Sczakiel et al. indicated that GLI3 variants linked to IPD can also occur in the ZFN and CS domains, challenging previous beliefs that such variants are confined to the C-terminal TAD domain, aligning with our findings in Family II [32].

A previous study proposed that GLI3 expression could demarcate between posterior and anterior hand anomalies. Specifically, GLI3 haploinsufficiency has been linked to the etiology of preaxial polydactyly, whereas postaxial polydactyly arises from abnormal truncations in the TAD [33]. In this study, Proband I, exhibited preaxial polydactyly traits, carried a truncating variant upstream of the ZFN in GLI3, likely leading to GLI3 haploinsufficiency. Conversely, Proband II, who displayed postaxial polydactyly, harbored a truncating variant in the CS domain of GLI3, potentially resulting in TAD functional deficiency. These observations align with findings from Bass et al. However, despite the variant in Proband III being associated with a TAD deficiency, the phenotype included both preaxial and postaxial polydactyly, highlighting gaps in current understanding of the relationship between *GLI3* variant locations and clinical manifestations. Future research is warranted to elucidate this association more comprehensively.

To investigate the genotype-phenotype correlation further, we analyzed 246 cases of polydactyly linked to *GLI3* variants (Fig. 3A). A predilection for variants in the C-terminal region of GLI3 was noted. Statistical analyses confirmed that the majority of polydactyly cases resulted from by loss-of-function *GLI3* variants (Fig. 3B), with 144 cases (approximately 59%) associated with GCPS (Fig. 3C), corroborating previous

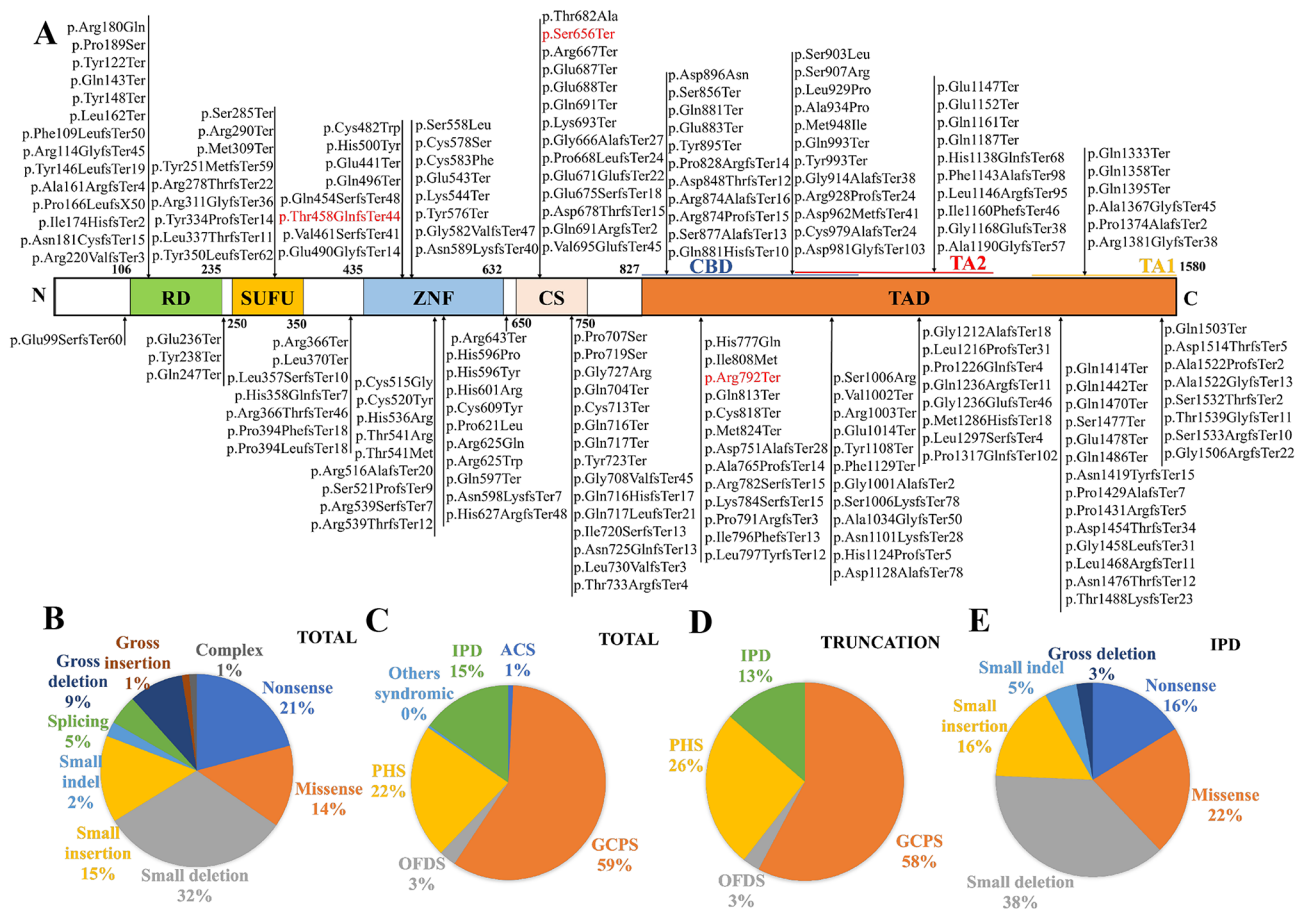


Fig. 3 Analysis of polydactyly related to *GLI3* reported in other studies. **(A)** Variants of the *GLI3* protein have been reported. **(B)** Types of *GLI3* variants involved in polydactyly. **(C)** Polydactyly types caused by *GLI3* variants. **(D)** Polydactyly types caused by *GLI3* truncation variants. **(E)** Types of *GLI3* variants involved in isolated polydactyly. RD = repressor domain; SUFU = SUFU site; ZNF = zinc finger domain; CS = cleavage site; CBD = CREB-binding protein (CBP) domain; TAD = transactivation domain; TA1 = transactivation domain 1; TA2 = transactivation domain 2

studies [13]. In this study, both Family I and III demonstrated features of GCPS. Furthermore, prior research estimated the incidence of IPD at 1.01 per 1000 live births [34], and our analysis revealed that a only 13% of IPD cases involved truncating *GLI3* variants (Fig. 3D). In this study, all patients exhibited *GLI3* truncations, yet the patients in Family II was diagnosed with IPD, whereas patients in Family I and III showed milder forms of GCPS, primarily affecting hands or feet. These findings suggest a tendency for milder phenotypic manifestations among *GLI3* truncation carriers in Hunan Province. Remarkably, a further statistical assessment of all IPD patients confirmed that loss-of-function *GLI3* variants are also a predominant pathogenic factor (Fig. 3E).

In this study, surgical procedures were performed on patients affected by polydactyly to remove the supernumerary fingers. This process may result in damage to the muscles and bones in the affected areas, impacting the patients' daily lives during the recovery period. Recent studies have identified multiple genes

associated with susceptibility to skeletal muscle injury and repair capacity [35–40], and significant differences in single nucleotide polymorphisms (SNPs) of these genes have been observed among populations with different levels of physical activity [41, 42]. These findings suggest that analyzing specific SNPs of susceptibility genes in patients could be beneficial in predicting their postoperative recovery capacity. For patients with high muscle susceptibility and low repair capacity, it may be advisable to consider therapeutic measures that promote muscle repair during surgery. For example, based on the ability of mesenchymal stem cells (MSCs) to differentiate into various mesenchymal tissues, applying MSCs to the injury site may significantly improve the biomechanical properties, structure, and function of the muscle postoperatively [43].

Conclusions

In conclusion, we identified a novel frameshift variant (c.1372del, p.Thr458GlnfsTer44), a novel indel variant (c.1967_1968delinsAA, p.Ser656Ter) and a previously

reported nonsense variant (c.2374 C>T, p.Arg792Ter) associated with polydactyly. This study not only elucidates potential mechanisms underlying GLI3-mediated polydactyly syndromes and broadens the variant spectrum of the *GLI3* gene, but also explores the correlation between genotype and phenotype.

Abbreviations

CBP	CREB-Binding Protein domain
CS	Cleavage Site
GCPS	Greig Cephalopolysyndactyly Syndrome
GLI3	GLI family zinc finger 3
GLI1	GLI family zinc finger 1
ZNF141	Zinc finger protein 141
IQCE	IQ domain-Containing Protein E
FAM92A	Family with sequence similarity 92, Member A
DACH1	Dachshund family transcription factor 1
MIPOL1	Mirror-Image polydactyly 1
PITX1	Paired-Like homeodomain 1
IPD	Isolated PolyDactyly
PHS	Pallister-hall syndrome
RD	Repressor domain
SHH	Sonic Hedgehog
SUFU	Suppressor of Fused site
TAD	Transactivation domain
WES	Whole-Exome Sequencing
ZFN	Zinc Finger domain
indel	insertion-deletion
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13018-024-04928-0>.

Supplementary Material 1

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Author contributions

RY.W. and Q.X. were responsible for Investigation, Visualization, Writing-original draft. SH.C. was responsible for Investigation. JY.J. was responsible for Methodology, Data curation. R.X. was responsible for Funding acquisition, Resources and Software, Supervision, Writing-review & editing. F.Y. was responsible for Conceptualization, Funding acquisition, Resources and Software, Supervision, Writing-review & editing. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study was approved (2023030444) by the Ethics Committee of Xiangya Hospital, Central South University, Changsha, China. We performed this study in accordance with the principles outlined in the Declaration of Helsinki. The patients/participants provided written informed consent to participate in the study.

Competing interests

The authors declare no competing interests.

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