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ORIGINAL ARTICLE

A novel variant of *GLI3*, p.Asp1514Thrfs*5, is identified in a Chinese family affected by polydactyly

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Abstract

Background: Polydactyly is a common congenital malformation characterized by the presence of supernumerary fingers or toes. In this case study, we sought to identify the causative pathogenic factor in a family from a northern region of China affected by non-syndromic postaxial polydactyly (PAP).

Methods: After recruiting a three-generation family with PAP, whole-exome sequencing was performed to identify the causative variant. In silico analysis and Sanger sequencing were used to validate the variant.

Results: We identified a novel heterozygous frameshift variant (NM_000168.6:c.4540delG, p.Asp1514Thrfs*5) in the transcriptional activator (TA1) domain of the *GLI3* gene.

Conclusion: The novel frameshift variant identified in this study further confirms the relationship between non-syndromic PAP and *GLI3* and extends the previously established mutational and phenotypic spectra of *GLI3*.

KEYWORDS

GLI3, PAP, polydactyly, Sanger sequencing, whole-exome sequencing

Yusi Wang and Xuguang Hao contributed equally.

1 | INTRODUCTION

Polydactyly is a class of congenital limb malformations that is most frequently observed at birth (Farrugia & Calleja-Agius, 2016) and can be classified into several types based on the position and morphology of the supernumerary digit(s) (Temtamy & McKusick, 1978). Among these, postaxial polydactyly (PAP; MIM# 174200) is characterized by additional digit(s) on the ulnar or fibular side of the extremities, whereas those with meso-axial polydactyly have additional fingers in the central part of the hand and preaxial polydactyly is characterized by digit(s) on the radial or tibial side of the extremities (Umair et al., 2018). Moreover, preaxial polydactyly can occur concurrently with other types of malformations, as described in Greig cephalonpolysyndactyly syndrome (GCPS; MIM 175700) and Pallister–Hall syndrome (PHS; MIM 146510) (Ito et al., 2018). PAP can be further sub-divided into postaxial polydactyly type A (PAPA) and postaxial polydactyly type B (PAPB) based on developmental status, the former of which is characterized by a well-formed redundant digit, whereas the latter manifests only as a minor protuberance (Malik, 2014). It has previously been established that polydactyly is primarily inherited in an autosomal dominant pattern, and that human polydactyly is associated with the GLI family zinc-finger 3 protein (*GLI3*; MIM*165240*)* (Al-Qattan et al., 2017; Umair et al., 2019; Verma & El-Harouni, 2015). The *GLI3* gene has been demonstrated to be one of the three GLI zinc-finger transcription factors that mediate the SHH-GLI3 pathway, and has been identified as playing an important role during embryogenesis, particularly with respect to formation of the neural tube, craniofacial structure, and limbs (Motoyama, 2006).

In this study, we examined a family with non-syndromic PAP caused by a novel variant (NM_000168.6:c.4540delG) of the *GLI3* gene based on a screening whole-exome variants.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance and sample characterization

For the purposes of this study, we recruited individuals from three generations of a Chinese family in Harbin, Heilongjiang Province, certain members of which are affected by polydactyly. Blood samples were collected from the three family members (designated II-1, II-2, and III-1), among whom, two are affected (II-2 and III-1) and the third unaffected (II-1). The diagnosis of congenital non-syndromic PAP in the family was confirmed by clinical and X-ray examinations. A pedigree diagram was drawn based on standard procedures and detailed interviews with family members. The study was approved by the Institutional Research Board of Harbin Medical University and all participating individuals provided their informed consent and gave permission for the publication of data and photographs.

2.2 | Whole-exome sequencing

To identify the pathogeny, whole-exome sequencing (WES), was performed commercially by Novogene Technology Limited-liability Company (Beijing, China), using genomic DNA extracted from peripheral blood of the proband (III-1).

Following Illumina paired-end protocols, the extracted genomic DNA was fragmented to an average size of 180–280 bp and used to generate DNA libraries. Exome capture was performed using an Agilent SureSelect Human All ExonV6 Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Sequencing of the genomic DNA using the Illumina Novaseq 6000 platform (Illumina Inc., San Diego, CA, USA) was performed commercially by Novogene Bioinformatics Technology Co., Ltd (Beijing, China) to generate 150-bp paired-end reads with a minimum coverage of 10× for ~99% of the genome (mean coverage of 100×). Having sequenced the DNA, basecall file conversion and demultiplexing were performed using bcl2fastq software (Illumina). The resulting FASTQ data were subjected to in-house quality control to remove low-quality reads, and were thereafter aligned to the reference human genome (hs37d5) using the Burrows-Wheeler Aligner (BWA) (Li & Durbin, 2009). Duplicate reads were marked using Sambamba tools (Tarasov et al., 2015). Annotation was performed using ANNOVAR (2017June8) (Wang et al., 2010). Annotations included minor allele frequencies from public control data sets, as well as deleteriousness and conservation scores, thereby enabling further filtering and assessment of the likely pathogenicity of variants.

2.3 | In silico analysis: Filtration and prioritization

WES was used to screen susceptibility variants in the family proband, with the following criteria being used to identify pathogenic variants: (1) a focus on genes associated with polydactyly that have been reported in the OMIM, HGMD, ClinVar, MalaCards, and PubMed databases; (2) priority accorded to heterozygous variants

given the predominant genetic tendency shown in the pedigree; (3) a variant minor allele frequency (MAF) of less than 0.1%; and (4) a pathogenic prediction. The accession numbers of different versions of the *GLI3* reference sequence are as follows: NG_008434.1; NM_000168.6, and NP_000159.3.

2.4 | Genomic DNA extraction and exome sequencing

Samples of peripheral blood were obtained from the three assessed family members (II-1, II-2, and III-1) and placed in qualified negative-pressure vacuum EDTA anticoagulant tubes. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, #69506, Dusseldorf, Germany) according to standard methods. The sequence of the *GLI3* gene was downloaded from the Ensembl Genome Browser (http://grch37.ensembl.org/Homo_sapiens/Info/Index), and specific primers used to amplify the identified variant (forward: 5′-AAGCTGGTTCATTCTCTATTTCA-3′, reverse: 5′-GATTTCCGTTGGTTGCAGTCTTTT-3′) were designed using PrimerSelect. Amplifications were performed using 40-μl reaction mixtures, containing 2 μl of template DNA, 2 μl each of the forward and reverse primers, 14 μl of DNA-free water, and 20 μl of GO Taq DNA polymerase (Promega, China). The PCR cycling conditions were as follows: pre-denaturation at 95°C for 3 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60.6°C for 30 s, and extension at 72°C for 30 s; with a final elongation at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis, and were then subjected to direct DNA sequencing using an Applied Biosystems 3730XL Genetic Analyzer (Foster City, USA) according to the manufacturer's instructions. To identify the genotype of each allele, the alleles were isolated in different clones using a pEASY-T1 Simple Cloning Kit (TransGen, China).

3 | RESULTS

3.1 | Clinical presentation

PAP was observed in affected individuals, as shown in the three-generation pedigree of the assessed family (Figure 1). Details of each of the family members are listed in Table 1. The proband (III-1) is affected by congenital non-syndromic polydactyly, for whom anomalous development was shown by radiographic examinations, and is characterized by a well-developed redundant digit on the right hand and bilateral feet. The left hand is also distinguished by an additional undeveloped nubbin. The

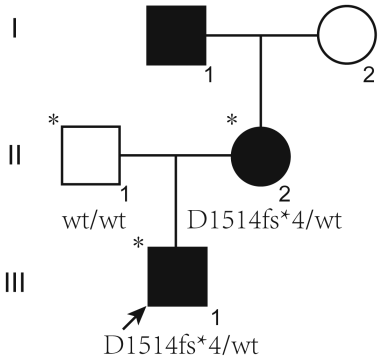

FIGURE 1 The pedigree of three generation of the Chinese family assessed in this study. Squares and circles represent males and females, respectively, and filled and unfilled symbols indicate affected and unaffected individuals, respectively. The proband is denoted by an arrow. An asterisk indicates that a peripheral blood sample collected from the individual is available

proband's mother (II-2) was born with congenital PAP of the bilateral hands and feet, and the proband's maternal grandfather (I-3) was also found to have congenital polydactyly.

3.2 | Identification of the causative variant

To screen for and identify pathogenic variants, we performed whole-exome sequencing of a sample of peripheral blood collected from the proband (III-1). Details of the genes relevant to the present study were sourced from the OMIM, HGMD, ClinVar, MalaCards, and NCBI databases, a list of which is presented in Table S1. In addition, by way of confirming pathogenicity, the MAF and predictions should meet the specified selection criteria, the associated filtering process of which is shown in Figure 2. This screening accordingly enabled us to identify a novel causative variant in the sequence of *GLI3* exon 15 (designated NM_000168.6:c.4540delG, NP_000159.3:p.Asp1514Thrfs*5), located in a highly conserved transcriptional activation (TA1) domain. This variant was not detected in any of the following databases: ExAC browser (http://exac.broadinstitute.org/), 1000 Genomes (http://browser.1000genomes.org), Exome Sequencing Project (https://evs.gs.washington.edu/EVS/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), and HGMD (http://www.hgmd.org). Moreover, we failed to detect the variant in any of the genome sequences obtained for 180 unaffected individuals (controls), who were a random sample of 1884 disease-free subjects examined in the Novo-Zhonghua project. This variant located in a highly conserved transcriptional activation (TA1) domain and the conservatism was also identified in the UCSC browser

TABLE 1 Characteristics of different members of the family screened in this study

Individual	Sex	Clinical features			
		Left hand	Right hand	Left food	Right food
I-1	M	PAP	PAP	PAP	PAP
I-2	F	.	.	.	
II-1	M	.	.	.	
II-2	F	PAP	PAP	PAP	PAP
III-1	F	PAPB	PAPA	PAPA	PAPA

Note: Unaffected parts are denoted by dots.

Abbreviations: PAPA, postaxial polydactyly type A; PAPB, postaxial polydactyly type B.

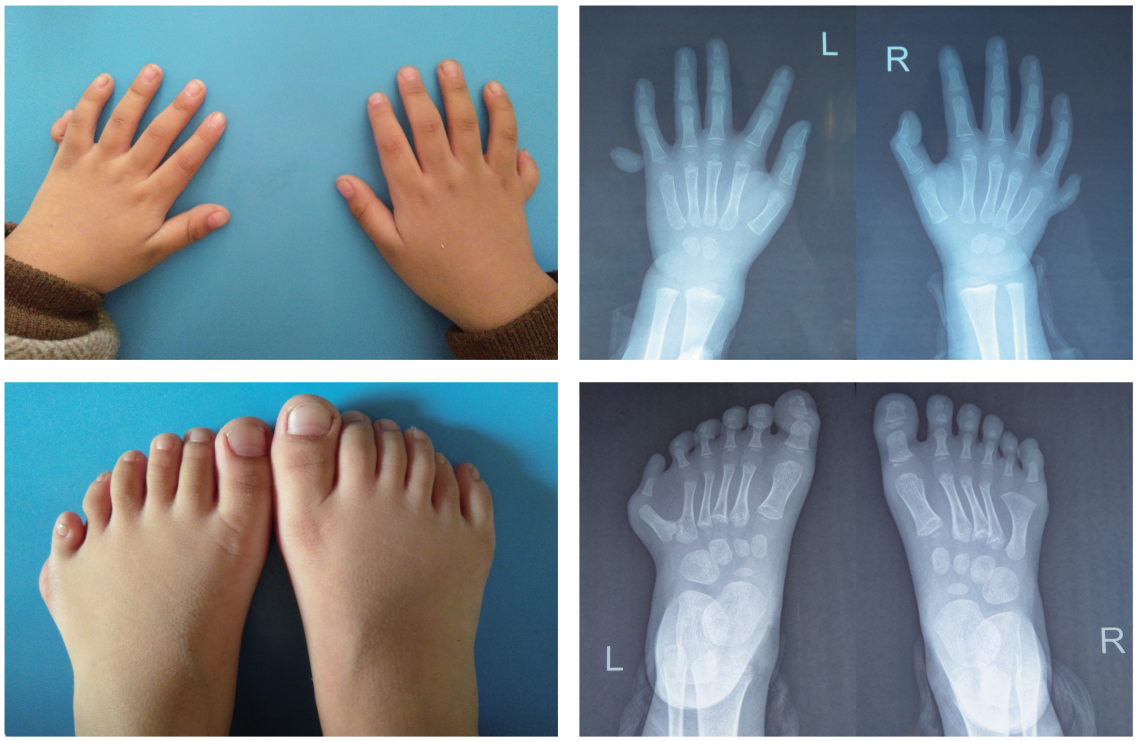

FIGURE 2 Clinical features of the proband. The proband was diagnosed with postaxial polydactyly type A in the right hand and bilateral feet, and postaxial polydactyly type B of left hand, as determined by x-ray examination

(http://genome.ucsc.edu/) and GeneCards (http://www.genecards.org/) databases. In addition, prediction results obtained using Mutation Taster (http://www.mutationtaster.org/) supports the identification of the variant, and predictions obtained using VarSome (https://varsome.com/) indicate that the identified variant is pathogenic with PVS1, PM2, and PP3.

Consistently, the results of Sanger sequencing indicated that the identified *GLI3* variant is correlated with disease phenotypes in all family members (Figure 3). Having thus identified the putative causal variant, we subsequently ligated the purified PCR products to a linearized T vector, as shown in Figure 4. Sequencing revealed both patients (II-2, III-1) to be heterozygotes with respect to the variant. In contrast, the proband's father (II-1) is an unaffected individual and carries a normal sequence. Thus, these observations provide evidence to indicate that the transmission of the disease is characterized by an autosomal dominant inheritance pattern and is consistent with individuals in the pedigree harboring heterozygous alleles.

3.3 | ACMG evaluation

To assess the pathogenicity of the *GLI3* variant, we followed the standards and guidelines of the American College of Medical Genetics (ACMG) (Richards et al., 2015). On the basis of Varsome results, loss-of-function is a known

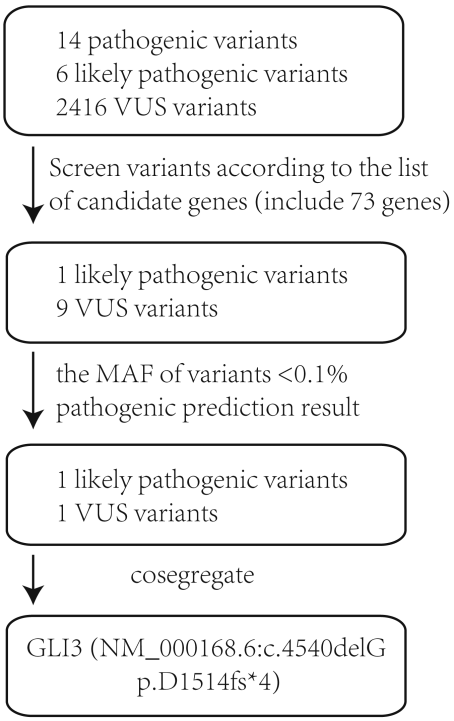

FIGURE 3 A flow diagram showing the process of causative variant identification

mechanism of the disease and a frameshift variant was identified as PVS1. The truncated variant (PM4) was located in a mutational hot spot (PM1) and absent from controls (PM2). Members of the family co-segregated (PP1) in an autosomal dominant pattern, which is consistent with PAP (PP4). In addition, the variant's deleterious effect is supported by computational evidence (PP3). Accordingly, there was strong evidence to indicate that the variant fulfilled the criteria of "pathogenic," with the very strong and three moderate (PM1, PM2, and PM4) and supportive (PP1, PP3, and PP4) evidence.

4 | DISCUSSION

In this study, we identified a novel frameshift variant of *GLI3* in a family affected by PAP. This variant co-segregated with a polydactyly phenotype in an autosomal dominant manner. The proband of the family is characterized by PAPA in the right hand and bilateral feet, and PAPB in left hand, and the proband's mother similarly suffers from polydactyly. On the basis of WES screening of the proband, we identified a novel variant of the *GLI3* gene (NM_000168.6:c.4540delG, NP_000159.3:p.Asp1514Thrfs*5).

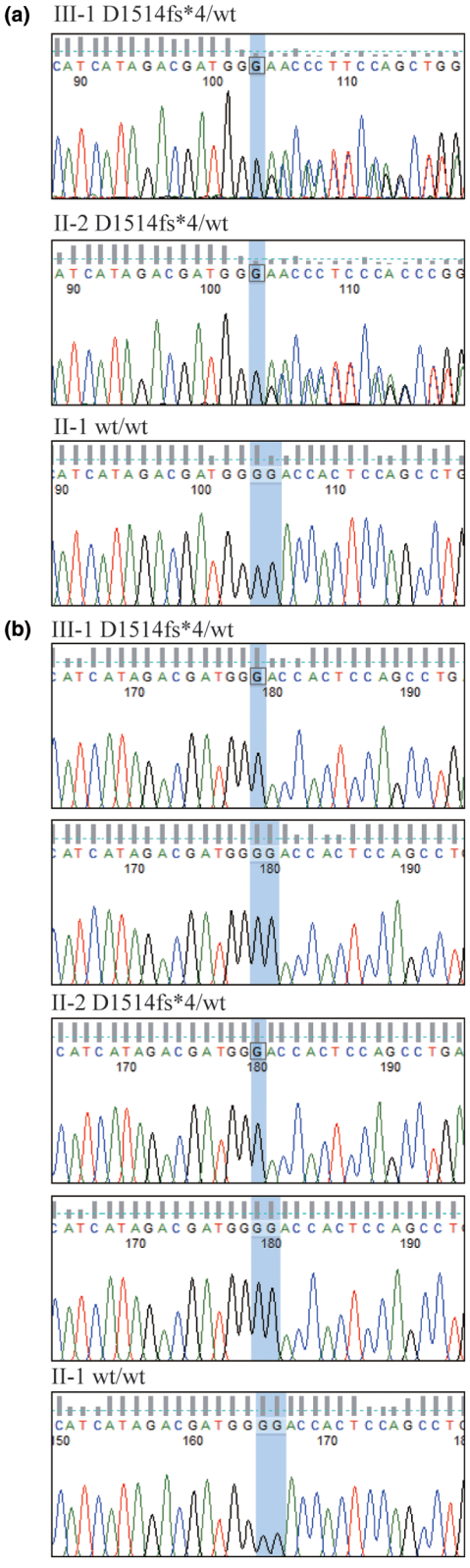

FIGURE 4 Sequencing analysis of *GLI3* (5′-3′). (a) A frameshift variant (c.4540delG) in the affected individuals (II-2, III-1) is indicated by highlights. The family member without polydactyly (II-1) does not carry this variant. (b) Sequencing analysis of the variant verified by T linear vector. The variant in patients II-2 and III-1 manifests as a heterozygote. The unaffected member (II-1) was confirmed to be homozygous for this locus, consistent with the NG_008434.1 reference sequence

It has previously been established that *GLI3* plays a dual role in the process of normal limb bud expression. The phosphorylated full-length form of GLI3 (GLI3A) activates the Sonic hedgehog (SHH) signaling pathway, whereas C-terminally truncated GLI3 (GLI3R) acts as a repressor. GLI3R antagonizes HAND2, thereby promoting the normal polarity and expression of SHH. It has been found that proteasomal cleavage leads to the truncation of GLI3R spanning exons 13 and 14, and in vertebrates, a balance between activation and inhibition is essential for normal embryonic development and limb formation (Al-Qattan & Al-Motairi, 2013; Hill et al., 2007). It has also been found that *GLI3*-deficient embryonic mice are characterized by abnormal limb development (Quinn et al., 2012; Veistinen et al., 2012). Collectively, these findings indicate that *GLI3* functions as a regulatory factor that contributes to determining the correct number and identity of digits during early limb development. In the present study, we validated the relationship between *GLI3* and the occurrence of polydactyly.

In previous studies, a robust relationship between *GLI3* and polydactyly has been reported (Malik, 2014; Patel et al., 2016, 2021). The *GLI3* gene comprises 15 exons that encode a 1580-amino acid protein containing a zinc-finger DNA binding domain, proteolytic cleavage site, CBP-binding region domain, and two transactivation domains (TA1 and TA2) (Kalff-Suske et al., 1999). The variant identified in the present study (p.Asp1514Thrfs*5) truncates the GLI3 protein from the TA1 domain, and interestingly, a large number of variants have previously been reported in this domain, as shown in Figure 5b. Several studies, particularly those focusing on syndromic polydactyly, have established a genotype–phenotype association in terms of the location of variants in a particular domain (Furniss et al., 2007; Johnston et al., 2005; Ship et al., 1999). Variants occurring between nucleotides 1998 and 3481 of the *GLI3* cDNA have been shown to be associated PHS, whereas variants downstream of nucleotide 1998 and upstream of nucleotide 3481 have been linked to GCPS (Johnston et al., 2010). Moreover, it has been established that the *GLI3* variants causing non-syndromic polydactyly are not restricted to any single-specific domain, although most published cases on isolated polydactyly indicate that a change in protein length is required. Furthermore, the type of variation may be phenotypically related. For example, PHS is caused by frameshift and nonsensical variants, whereas GCPS can be associated with most types of variants. However, the location and

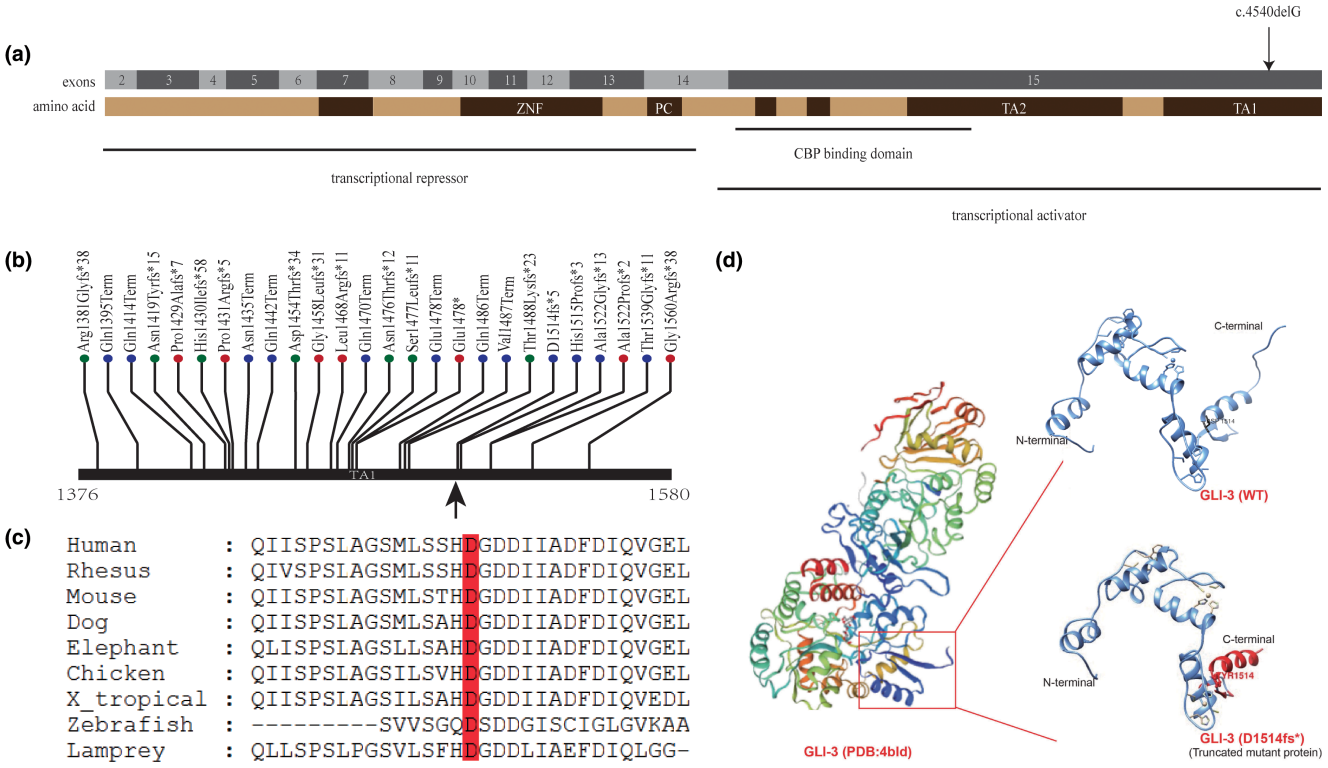

FIGURE 5 Analysis of the variant identified in the assessed family. (a) Analysis of *GLI3* motifs revealed that the variant is located in the TA1 transactivation domain and that the GLI3 protein is truncated by the frameshift mutation. (b) All variants of the TA1 domain are shown in the graph, with the variant identified in the present study being indicated. (c) Analysis of conserved amino acid residues among different mammals indicated that this variant is associated with disease. (d) The predicted structure of proteins. The length of the variant protein is clearly altered

type of non-syndromic PAP mutations have yet to be sufficiently determined (Johnston et al., 2005). Accordingly, identification of a large number of non-syndromic polydactyly cases caused by *GLI3* mutations will be necessary to further confirm the association and establish a genotype–phenotype relationship.

On the basis of the stringent criteria applied in the present study, the variant we identified can be classified as "pathogenic," and its physical effects are apparent. In addition to in silico analysis, both upstream and downstream variants lead to disease. The variants associated with *GLI3* pathogenesis are listed in Table S2. McDonald-McGinn et al. (2010) have reported a pathogenic variant (c.4542-4545delCCAC) located two bases downstream of the variant identified in the present study, which was found to be associated with features such as trigonocephaly and metopic craniosynostosis consistent with GCPS, whereas the individuals screened in our research are affected by non-syndromic polydactyly. In this regard, it is necessary to take into account variant penetrance. Other patients with *GLI3* TA1 domain variants have been found to have different phenotypes, including polydactyly, and whereas the proband reported in the present study is characterized by PAPA in the left hand and PAPB in the right, other studies have found that patients invariably have limb deformities.

The proband we describe herein has shown that this variant can lead to different types of PAP including PAPA (right hand and bilateral feet) and PAPB (left hand), which are distinguished by the integrity of excrescent digit. Collectively, the findings of the aforementioned studies serve to highlight that variants of the *GLI3* gene, particularly those that modify the TA1 domain, can potentially contribute to different types of polydactyly. In the present study, we established an association between this domain and PAP, although its association with other symptoms remain to be elucidated.

In conclusion, we identified a novel *GLI3* variant, c.4540delG (p.Asp1514Thrfs*5), in members of a Chinese family with postaxial polydactyly. Our characterization of this novel truncated variant expands our current understanding of the phenotypic spectrum of developmental malformations, and provides a reference for gaining further insights into the underlying genetic mechanisms.

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AUTHOR CONTRIBUTIONS

YW and MH: Drafted the manuscript and analyzed data. XJ, WJ, XZ, and LX: Contributed to exome data analysis and interpretation and variant confirmation. XH and YL: Contributed to clinical data collection and analysis. EJAG and SY: Revised the manuscript and organization of data. SF, WS, and JB: Manuscript editing and revision. JW: Supervised the work. All authors have read and approved the final manuscript.

ETHICAL STATEMENT

The present study was approved by the Institutional Research Board of Harbin Medical University, and all participants provided written informed consent.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

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