Two nonsense GLI3 variants are associated with polydactyly and syndactyly in two families by affecting the sonic hedgehog signaling pathway

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Abstract

Background: Polydactyly and syndactyly are congenital limb deformities, segregating in an autosomal-dominant fashion. The variants in the GLI3 gene are closely related to congenital limb malformations. However, the causes underlying polydactyly and syndactyly are not well understood.

Methods: We conducted a whole-exome sequencing on two four-generation Chinese families with polydactyly and syndactyly. Then c.2374C>T and c.1728C>A mutant plasmids were transfected to HEK293T cells and mice limb bud cells to explore the functional consequences of these variants. Western blot and real-time quantitative PCR were used to analyze the expression of GLI3 and Shh.

Results: In these two families, the known GLI3 variant (NM_000168.6:c.2374C>T) and the novel GLI3 variant (NM_000168.6:c.1728C>A) contributed to polydactyly and syndactyly. Additionally, the GLI3 c.2374C>T mutant plasmid led to truncated GLI3 protein, and the GLI3 c.1728C>A mutant plasmid led to degraded GLI3 protein. Simultaneously, we demonstrated that the GLI3-mutant plasmids led to decreased Shh expression in mice limb bud cells.

Conclusion: We demonstrated that the novel GLI3 variant (c.1728C>A) and known GLI3 variant (c.2374C>T) contributed to the malformations in two four-generation pedigrees with polydactyly and syndactyly by affecting SHH signaling.
1 INTRODUCTION

Polydactyly is the most frequently observed congenital hand malformation, with a prevalence of approximately two per 1000 live births (Anderson et al., 2012). Syndactyly is characterized by a cutaneous or osseous fusion of adjacent digits, which is one of the most common forms of all congenital hand deformities with an incidence of around 1 in 2000 live birth (Jordan et al., 2012). Polydactyly and syndactyly both segregate in an autosomal-dominant fashion.

Human limb bud development is a highly complex and conserved pattern, which is the basis of digits development (Petit et al., 2017). Sonic hedgehog (SHH) signaling pathway acts an important role to regulate the digits pattern in limb formation, specifying anteroposterior pattern in developing limbs. And glioma-associated oncogene homolog 3 (GLI3, OMIM: 165240), a zinc finger transcription factor, is one of the most important downstream factors in the SHH signaling pathway (Al-Qattan et al., 2017; Villavicencio et al., 2000). GLI3 has two kinds of forms, full-length (GLI3-FL) and repressor (GLI3-R) form. When the SHH signaling pathway is absent, GLI3 is phosphorylated and partially degraded or converted to GLI3-R which leads to repression of the SHH signaling pathway. When the SHH signaling is present, GLI3-FL specifically targets the promoter of GLI1 to regulate SHH signaling pathway-associated genes (Matissek & Elsawa, 2020).

The role of GLI3 in limb development is widely studied in two kinds of human congenital malformations including Pallister–Hall syndrome (PHS) and Greig cephalopolysyndactyly syndrome (GCPS). Pallister–Hall syndrome is a pleiotropic autosomal-dominant disorder comprising visceral malformations, pituitary dysfunction, central polydactyly, and hypothalamic hamartoma (Hall, 2014). Johnston et al. (2005) reported that GLI3 mutations that predicted a truncated functional repressor protein caused Pallister–Hall syndrome. Greig cephalopolysyndactyly syndrome is characterized by preaxial and postaxial polydactyly, variable syndactyly, scaphocephaly, frontal bossing, and hypertelorism (Biesecker, 2008). Using a candidate gene approach to test the possible implication of the GLI3 gene in this disorder, Vortkamp et al. (1991) demonstrated that two of three translocations were associated with GCPS by interrupting the GLI3 gene. These studies demonstrate that the variants in the GLI3 gene are closely related to congenital digit malformations.

In this study, we explored the genetic basis of polydactyly and syndactyly in two four-generation Chinese families. Using whole-exome sequencing followed by Sanger sequencing, we identified a novel heterozygous nonsense variant c.1728C>A (p.Y576X) in GLI3 that segregated with the disease phenotype within Family 2. We also identified a known nonsense variant c.2374C>T (p.R792X) using whole-exome sequencing in Family 1, and this point variant was previously reported to be associated with polydactyly and syndactyly (Furniss et al., 2007). In vitro, we observed that these two variants led to truncated or degraded GLI3 protein. Meanwhile, the Shh expression levels were decreased when mice limb bud cells were transfected with mutant plasmids, which resulted in limb malformations.

2 MATERIALS AND METHODS

2.1 Subjects

Two four-generation Han Chinese pedigrees with polydactyly and syndactyly were identified. In Family 1, 17 family members (6 affected members and 11 normal members) participated in this study. In Family 2, 34 family members (11 affected members and 23 normal members) participated in this study. Polydactyly and syndactyly were diagnosed based on X-ray examination, physical examination, and family history. DNA samples were extracted from peripheral blood using QIAamp DNA blood mini kits (Qiagen, Germany).

2.2 Genetic studies

Exome sequences were enriched with an Agilent SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA). Sequences were generated on a HiSeq PE150 (Illumina). Base-calling was performed, and raw-sequencing read files were generated in FASTQ format. Subsequently, we aligned the sequenced reads to the reference human genome (NCBI Build 37, hg19) and performed the annotation using SeattleSeq Annotation 150 (version 9.10). In this study, the GenBank reference sequence and version number is NC_000007.13.

We then used various databases to predict and filter all variants, such as the database of single nucleotide polymorphisms (dbSNP), 1000 genomes, SIFT, Polyphen-2, and Mutation Taster. All variants included in the most recent version of the National Center for Biotechnology Information...
dbSNP were excluded. Low-frequency frameshift and truncating variants (minor allele frequency <0.001) in any genes were considered potentially pathogenic. Finally, we chose variants that were shared by affected individuals but not present in the unaffected individual.

2.3 | Genotyping

Genomic DNA was isolated from 200 μl of blood per subject and was diluted to a final concentration of 15–30 ng/μl for genotyping assays. Polymorphism-spanning fragments were amplified using PCR and genotyped using the MassArray system (Sequenom, San Diego, CA) with primers of c.17280C>A (Fwd: TGACCAGTAGGTGGCAGTT, Rev: GCTACATCTGAATCCCAATAAA), prepared by the Beijing Genomics Institute (Shenzhen, China), as described previously (Gao et al., 2017).

2.4 | Plasmid constructs and transfection

Full-length, wild-type (WT) human GLI3 cDNA was amplified (Fwd: 5′-CGCAAATGGGCTGTCGTCG-3′, Rev:5′-TAGAAGGCCACAGTCGAGG-3′), and GLI3 cDNA was cloned into H302 pcDNA3.1(+) vector (Obio corporation, Shanghai, China). Mutagenesis of GLI3 (to introduce variants encoding the p.R792X and p.Y576X) was performed using a QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene, Santa Clara, California). HEK293T cells and mice primary limb bud cells were seeded in six-well plates and transiently transfected with plasmids using Lipofectamine 3000 (Invitrogen). Cells were harvested 48 hr after transfection.

2.5 | Cells culture

Primary mice limb buds are isolated from embryonic day 11.5 under a stereo light microscope and digested with 0.25% trypsin for 10 min at 37 °C. Limb bud cells were seeded in a six-well plate and cultured at 37 °C with 5% CO₂. DMEM (Gibco) supplemented with 1% penicillin–streptomycin solution (Gibco), and 10% fetal bovine serum (PAN) was used as the culture medium. HEK293T cells were cultured in DMEM (Gibco) high glucose containing 10% fetal bovine serum (Gibco) at 37 °C with 5% CO₂.

2.6 | Western blot analysis

The proteins of HEK293T cells were extracted with RIPA buffer (Beyotime) containing 1% phosphatase and protease inhibitors (Bimake), followed by measurement of protein concentrations using a BCA protein assay (Beyotime). After boiling denaturation, the protein samples (20 μg/sample) were subjected to 7.5% SDS-PAGE and then transferred to nitrocellulose membranes (Pall). After being blocked with 5% skim milk for 1 hr at room temperature, the membranes were probed with primary antibody against Flag (101274-MM05, Sino Biological), HA (100028-MM10, Sino Biological) at 4 °C overnight and then washed three times with TBS-T before incubation with antimouse secondary antibody (7076, Cell Signaling Technology) for 1 hr at room temperature. Color development was performed using an ECL chemiluminescence detection kit (Beyotime, Shanghai, China), and images of protein bands were captured using a gel imager (GE, ImageQuant Las4000mini).

2.7 | Real-time quantitative PCR

Total RNA was extracted from mouse limb bud cells with RNAiso Plus reagent (Roche). cDNA was synthesized using 500 ng of total RNA and a Prime-Script RT reagent kit with a gDNA Eraser (Takara) following the manufacturer’s instructions. Quantitative PCR was performed to amplify the cDNA on a Bio-Rad CFX96 Real-Time PCR System using TB Green Premix Taq II (Takara, Japan) and primers (Fwd: AAAGCTGACCCCTTTAGCCTA; Rev: TTCGGAGTTTCTTG TGATCTTCC). Relative expression levels were calculated by the 2−ΔΔCt method. GA0PDH served as the internal control for normalization.

2.8 | Statistical analysis

Statistical analysis was performed using the statistical software SPSS13. Results are expressed as mean ± standard deviation (s.d.) and analyzed for significant differences using analysis of variance (ANOVA) and Student’s t test. p < .05 was considered significant (*p < .05, **p < .01, ***p < .001).

3 | RESULTS

3.1 | Clinical assessment of two families with polydactyly and syndactyly

Two four-generation families with polydactyly and syndactyly from Wuxi Ninth People’s Hospital Affiliated to Soochow University were recruited for the study. No history of consanguineous marriage in this family was
recorded according to the senior family members’ statements. In these two families, the family history of the disorders was consistent with an autosomal-dominant pattern of inheritance. Phenotypic variability was observed among affected individuals.

The clinical assessment of five affected members in Family 1 was summarized in Figure 1 and Table 1. The clinical assessment of six affected members in Family 2 was summarized in Figure 2 and Table 2. The depth and coverage of whole-exome sequencing in Family 2 were presented in Table 3. According to the clinical assessment of these two families, we observed phenotypic variability among affected individuals which was consistent with previous research. Except for polydactyly and syndactyly, there were no other abnormalities of the shape of the head, maxillofacial malformation, brain imaging, distance of the inner canthal, interpupillary distance, height or limb length, organa genitalia, anus, and throat (epiglottis) observed in these two families. All the patients in Family 1 were diagnosed as synpolydactyly-1 (OMIM: 186000), all the patients in Family 2 were diagnosed as synpolydactyly-3 (OMIM: 610234).

**FIGURE 1** Clinical features of the affected members in Family 1. (a) Pedigree structure of a four-generation Chinese family with complex digital anomalies. Squares and circles denote males and females. Filled shapes indicate affected members. The arrow denotes the proband. Members marked with * are the participants in this study. (b) Clinical features of the proband (IV:2) and affected individuals (IV:1/III:2/III:3/II:2) are listed in Table 1
Identification of point variants in the GLI3

To identify variants that predispose to syndactyly and polydactyly, whole-exome sequencing was initially performed on affected individuals and healthy members of these two pedigrees. In Family 1, IV:1, IV:2, III:1, III:2, III:3, II:1, and II:2 were subjected to whole-exome sequencing. In Family 2, IV:2, III:10, and III:11 were subjected to whole-exome sequencing. As previously reported (Zhou et al., 2019), we annotated and filtered variants and kept variants that were novel in dbSNP. Polyphen-2, Mutation Taster, and Genomic Evolutionary Rate Profiling (GERP) were then used to predict the potential functional effects of these mutations.

In Family 1, we identified 10 candidate single nucleotide variations (SNVs), in which c.C2374T (Refseq NM_000168) in the GLI3 (MIM:165240) has been reported to be associated with syndactyly and polydactyly (Table 4) (Furniss et al., 2007).
three candidate SNVs. Then by using Sanger sequencing, we excluded the SNV on **WDR34** and **FREM2**, because **GLI3** c.C1728A (Refseq NM_000168) turned out to be the only one that cosegregated with disease phenotypes in this family (Table 5). The location of GLI3 p.R792X and p.Y576X were presented (Figure 3a). The GLI3 p.Y576X was located in the ZNF domain and the GLI3 p.R792X was located outside the ZNF domain. According to American College of Medical Genetics and Genomics (ACMG) standards and guidelines, the new variant in Family 2 was classified as pathogenic. According to the Mutation Taster, the GLI3 p.R792X and p.Y576X locus both showed conservation between species (Figure 3b).

### Table 2: The symptoms of digits abnormalities in six affects members of Family 2

| Family and patient’s ID | Lower limb | Upper limb | Clinical diagnosis |
|-------------------------|------------|------------|--------------------|
|                         | Preaxial polydactyly | Syndactyly | Postaxial polydactyly | Preaxial polydactyly | Syndactyly | Postaxial polydactyly |                     |
| IV:2                    | Bil         | Bil (1–3)  | —                   | Bil                   | Bil (3–4)  | —                   | SPD 3               |
| IV:5                    | Bil         | Bil (1–2)  | —                   | —                     | —          | —                   | SPD 3               |
| IV:6                    | —           | L (1–2), R (1–3) | —                   | Bil                   | Bil (3–4)  | —                   | SPD 3               |
| III:10                  | —           | Bil (1–2)  | —                   | —                     | L (3–4)    | —                   | SPD 3               |
| III:14                  | L           | —          | —                   | —                     | L (3–4)    | —                   | SPD 3               |
| III:16                  | Bil         | —          | —                   | —                     | —          | —                   | SPD 3               |

**Abbreviations:** Bil, bilateral; R, right; L, left; SPD, synpolydactyly.

### Table 3: Depth and coverage of whole-exome sequencing in Family 2

| Sample | IV:2                  | III:11                 | III:10                 |
|--------|-----------------------|------------------------|------------------------|
| Total reads | 89,485,646          | 107,931,572            | 96,863,472             |
| Duplicate | 21.38%                | 24.54%                 | 22.22%                 |
| Mapped | 99.98%                | 99.98%                 | 99.98%                 |
| On target rate | 80.91%               | 83.57%                 | 83.18%                 |
| On target mean coverage 1X | 112.65             | 134.82                | 123.73                |
| On target mean mapping quality | 57.90             | 57.82                 | 57.84                 |
| Fraction of target covered with at least 1x | 99.12%             | 99.46%                | 99.39%                |
| Fraction of target covered with at least 10x | 94.00%             | 95.11%                | 94.60%                |
| Fraction of target covered with at least 50x | 71.74%             | 76.04%                | 74.06%                |

### Table 4: Detailed information for GLI3 c.C2374T after annotation

| cDNA | GLI3 c.C2374T |
|------|---------------|
| Amino acid change | GLI3 p.R792X |
| In dbSNP or not | YES |
| Function GVS | Nonsense mutation |
| ConsScore GERP | 3.7 |
| phastCons20way_mammalian | 0.956 |
| phyloP20way_mammalian | 0.079 |
| Mutation Taster | 1(Disease-causing-automatic) |

**Note:** GenBank reference sequence and version number: NC_000007.13.

### Table 5: Detailed information for GLI3 c.C1728A after annotation

| cDNA | GLI3 c.C1728A |
|------|---------------|
| Amino acid change | GLI3 p.Y576X |
| In dbSNP or not | NONE |
| Function GVS | Nonsense mutation |
| ConsScore GERP | −3.75 |
| phastCons20way_mammalian | 0.974 |
| phyloP20way_mammalian | −0.738 |
| Mutation Taster | 1(Disease-causing-automatic) |

**Note:** GenBank reference sequence and version number: NC_000007.13.
To further explore the functional consequences of these variants, c.2374C>T and c.1728C>A mutant plasmids and GLI3 wild-type plasmid were constructed. To validate these two nonsense mutations, we transfected these plasmids into HEK293T cells separately. Compared with the wild-type GLI3, the Western blotting analysis showed that the GLI3 p.R792X protein was shorter (Figure 3c), whereas the GLI3 p.Y576X was undetectable, maybe due to the degradation of GLI3 p.Y576X protein (Figure 3c). To investigate whether these two GLI3 nonsense variants affected SHH signaling pathway, we then transfected the

**3.3 Functional studies on the GLI3 c.C2374T and c.C1728A variants**

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wild-type and mutant plasmids into mice limb bud cells separately. The qPCR analysis showed that c.C2374T and c.C1728A variants in GLI3 led to decreased Shh expression compared with the wild-type GLI3 (Figure 3d). In conclusion, these two GLI3 nonsense mutations may result in syndactyly and polydactyly by affecting the SHH signaling.

4 | DISCUSSION

In the presence of SHH signaling, GLI3 is maintained as GLI3-FL form which induces its downstream target genes, such as Patched1 (Ptch1). However, in the absence of SHH signaling, GLI3 is either phosphorylated, ubiquitinated, and then partially degraded. The short form of GLI3 is GLI3-R, which represses the transcription of its downstream target genes (Xiang et al., 2020). The mutations in GLI3 are closely related to congenital limb malformations such as GCPS and PHS. Individuals suffering from GCPS are characterized by preaxial polysyndactyly of the feet, postaxial polysyndactyly of the hands, and macrocephaly. In GCPS, mutations in GLI3 include missense mutations, splicing mutations, deletions, insertions, and translocations. These mutations can occur throughout the GLI3 protein and disrupt the balance between GLI3-R and GLI3-FL, resulting in the lack of negative regulation of SHH signaling (Johnston et al., 2010; Abdullah Yousaf et al., 2019). PHS is clinically identified by mesoaxial polydactyly and hypothalamic hamartoma. In PHS, mutations in GLI3 include frameshift and nonsense mutations, which lead to a truncated version of GLI3 (691 AA long). This truncated GLI3 showed inhibitory functions similar to GLI3-R in the context of SHH signaling (Böse et al., 2002; Kang et al., 1997). These studies demonstrate the importance of GLI3 in limb development.

Shh is expressed at the posterior margin of the limb buds in vertebrates, specifying an anteroposterior pattern in developing limbs by stimulating the proliferation of mesenchyme and regulating the anteroposterior length of the apical ectodermal ridge. As we previously reported, ectopic anterior expression of SHH in the hindlimbs led to preaxial polydactyly in ZRS g.101779T>A homozygous mice models (Xu et al., 2020). Yoshiyuki et al. demonstrated that a severe lack of SHH signaling led to the polydactyly, syndactyly, and brachydactyly in homoygote hereditary multiple malformation mutant (hmm−/−) mouse embryos. In the hmm−/− limb bud, Shh was restricted to a more proximal region of the forelimb bud than in the hmm+/− limb bud. Besides Gli1, Ptch1, Ptch2, and Bmp2 which are known to be downstream target genes of SHH signaling showed decreased expression (Matsubara et al., 2016). It is frequently described that altered Shh signaling is involved in congenital digit malformations under clinical conditions. Under the delicate regulation between GLI3 and SHH signaling, the limb bud revolves into normal digits.

In our study, we transfected GLI3 c.2374C>T and c.1728C>T A mutant plasmids into HEK293T cells. The GLI3 c.C2374T variant led to truncated GLI3 protein, and the GLI3 c.C1728A variant led to degraded GLI3 protein. We assumed that this kind of truncated or degraded GLI3 protein may affect the SHH signaling, resulting in polydactyly and syndactyly phenotype in these two families. Then we transfected the mutant plasmids into the mouse limb bud cells to detect the changes in Shh expression. Surprisingly, we found that mutant plasmids led to decreased Shh expression. Surprisingly, we found that mutant plasmids led to decreased Shh expression. The dysfunction of SHH signaling was responsible for the polydactyly and syndactyly in these two families.

5 | CONCLUSION

We demonstrated that the novel GLI3 variant (c.1728C>A) and known GLI3 variant (c.2374C>T) contributed to the malformations in two four-generation pedigrees with polydactyly and syndactyly. The GLI3 c.C1728A variant led to truncated GLI3 protein and the GLI3 c.2374C>T variant led to degraded GLI3 protein. Both the mutant plasmids led to decreased Shh expression, which resulted in limb malformation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS’ CONTRIBUTIONS

RYJ and ZTF conceived the idea and provided scientific support and professional guidance; SXF and ZS designed the project, conducted experiments, analyzed the data, performed statistical analyses, and wrote the manuscript; ZX revised the manuscript.

ETHICAL COMPLIANCE

The study was approved by the ethics committee of the Wuxi Ninth People’s Hospital Affiliated to Soochow University. All experimental protocols were approved by the ethics committee of the Wuxi Ninth People’s Hospital Affiliated to Soochow University and were carried out in accordance with the approved guidelines. Each individual
in these two families undergoing the genetic test was adequately informed regarding the benefits and risks of the test and signed the consent forms.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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