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IDENTIFICATION OF A RARE *GLI3* VARIANT ASSOCIATED WITH UNILATERAL THUMB POLYDACTYLY

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ABSTRACT

Polydactyly is a congenital anomaly marked by supernumerary digits, resulting in an excess number of fingers or toes. Numerous investigations have elucidated the critical role of genetic factors in determining the presence of non-syndromic polydactyly. Nevertheless, a comprehensive understanding of the underlying signaling pathway responsible for this disorder remains incomplete. In this study, we present a thorough analysis of a unique unilateral polydactyly phenotype in the thumb of a 6-year-old male proband, employing exome sequencing. The investigation revealed a rare heterozygous substitution variant (NM_000168.6:c.1384A>G; p.Lys462Glu) in the *GLI3* gene, a critical factor associated with polydactyly. Sanger sequencing confirmed the paternal inheritance of this variant. Notably, the amino acid change demonstrated evolutionary conservation, emphasizing its potential functional significance. Our findings contribute novel insights into the genetic underpinnings of polydactyly, highlighting the significant role of *GLI3* in limb development. This discovery expands our understanding of the broader implications of *GLI3* mutations in congenital limb abnormalities, paving the way for further investigations in this field. From a genetic perspective, unraveling the intricacies of polydactyly at the molecular level opens avenues for comprehending the broader genetic landscape of limb malformations and their underlying mechanisms.

Keywords: congenital anomaly, exome sequencing, *GLI3*, thumb polydactyly, zinc finger protein.

INTRODUCTION

Polydactyly, also called hyperdactyly or hexadactyly, is a congenital anomaly within the domain of limb formation pathology. The clinical manifestations of this disorder are systematically classified into three distinct groups based on the spatial orientation of the supernumerary digit: preaxial (radial), central, or postaxial (ulnar) (Ishigaki *et al.*, 2019). Variability in the anatomical localization of the duplicated digit is notably discernible across individuals with diverse racial or ethnic

backgrounds, since postaxial polydactyly is more frequently observed in the African population and preaxial polydactyly is more common in Caucasian patients (Singer *et al.*, 2014). The incidence of polydactyly ranges from 0.3 to 3.6 per 1000 live births, depending on different populations (Umair *et al.*, 2018). Polydactyly can contribute to other congenital defects as part of a syndrome (syndromic polydactyly) or occur as an isolated disorder (non-syndromic polydactyly). Syndromic types of congenital hand deformities are associated with other characteristics such as skull and facial deformities or slow mental development (Nguyen & Hoang, 2021).

Although polydactyly can be associated with various genetic factors, with multiple genes being involved in its development. However, there is no specific gene that is universally recognized as the sole cause of thumb polydactyly, suggesting that polydactyly, particularly its outcome and variability in phenotype, is influenced by a complex interplay of genetic factors and gene-gene interaction networks (Bubshait, 2022). Non-syndromic polydactyly commonly demonstrates autosomal dominant inheritance characterized by variable penetrance. In humans, the genetic landscape of this condition comprises a repertoire of at least ten loci and causative genes, notably *GLI3*, *ZNF141*, *MIPOL1*, *PITX1* and other genes (Deng *et al.*, 2015).

In this study, we investigated a Vietnamese trio family with two affected members, a father and his son, both presenting with polydactyly. The exome of the proband was sequenced to identify potential causative mutations associated with the condition.

MATERIALS AND METHODS

Patient description

The trio family under investigation belonged to the Kinh ethnic group, and lived in Ha Nam province, Vietnam. Blood samples were procured at the Department of Orthopedics, Vietnam National Children's Hospital, and preserved in EDTA-containing tubes at -20 °C. All participants were provided with comprehensive information regarding the study and subsequently signed informed consent forms before contributing their samples. The study was carried out in compliance with the Declaration of Helsinki and was approved by the Council for Ethics in Biomedical Research of the Vietnam National Children's Hospital (No. 564/BVNTW-VNCSKTE).

Whole-exome sequencing

Whole-exome sequencing (WES) was conducted on the NovaSeq 6000 system (Illumina, USA) for the proband. DNA libraries were prepared utilizing Exome Capture SureSelectXT v7 libraries, featuring 151 bp paired-end reads. The resulting sequencing reads were aligned to the human reference genome (UCSC GRCh38) using the Burrows-Wheeler Aligner (BWA-0.7.17). Variant calling procedures were executed using the Genome Analysis Toolkit (GATK v 4.1.2.0), involving the identification of variations such as single-nucleotide variants (SNVs) and small insertions or deletions (INDELs), in the sequenced DNA compared to the reference genome. The identified variants were interpreted and annotated using ANNOVAR.

Confirmation by Sanger sequencing

Verification of the *GLI3* gene variation obtained from the exome of the proband and other family members was conducted through Sanger sequencing. A pair of primers (FP: 5'–GTTACCGGGTCTTGGAAGGG-3'; RP: 5'-CAGGGCAGCAAAGTTTGACC-3') was employed to amplify the targeted genomic locus and its adjacent regions using the PCR Mastercycler nexus GSX1 (Eppendorf). Subsequently, the purified PCR products were subjected to sequencing using the ABI Big Dye Terminator v3.1 Sequencing Standard Kit. The obtained sequencing data were compared to the reference *GLI3* sequence (NM_031275.4) using Chromas 2.6.6 (Technelysium Pty Ltd).

RESULTS

The proband was a 6-year-old male at the time of sample collection, and he was the sole son in the family participating in the study. The distinctive phenotype observed was unilateral polydactyly of the thumb in the right hand (Figure 1A). Notably, both the father and the son displayed thumb polydactyly on one hand; however, the father had undergone surgical intervention, precluding visualization of the polydactyly pattern. Conversely, the mother exhibited a normal hand morphology (Figure 1B).

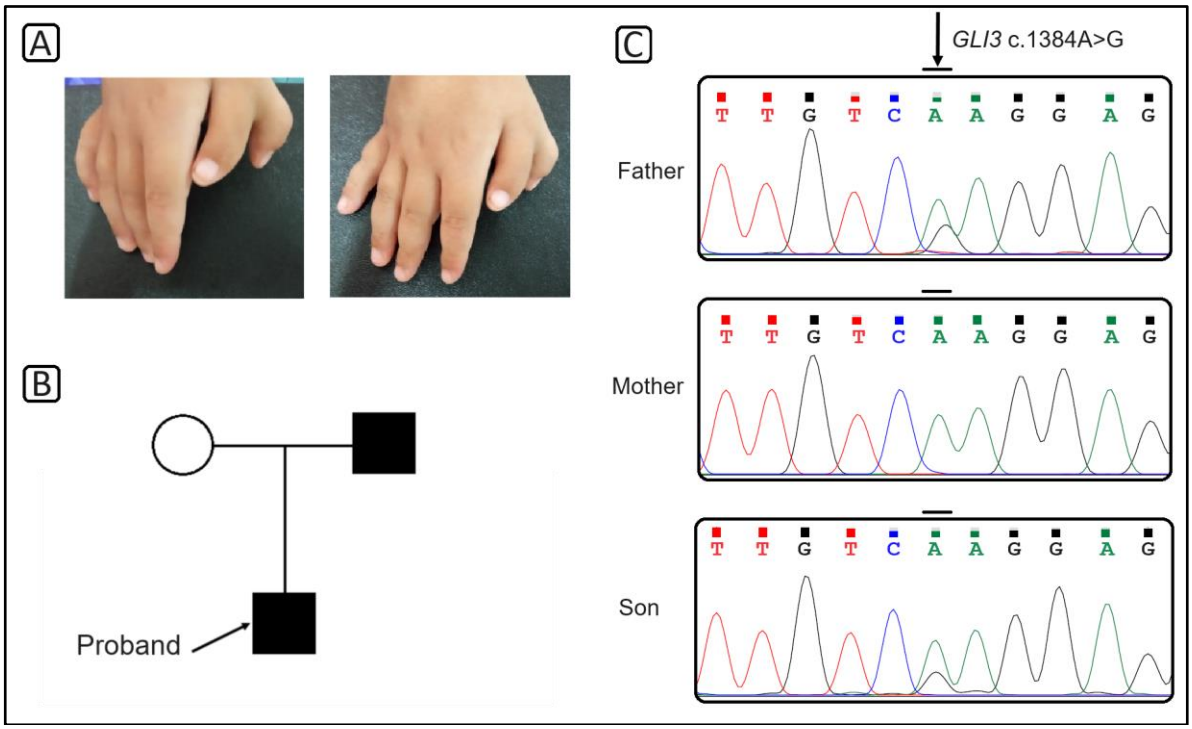

Figure 1. (A) Phenotypic manifestation of polydactyly in the proband; (B) Pedigree representation of the affected family; (C) Sanger sequencing outcomes illustrating genetic variations in the patient family pedigree.

Exome sequencing was employed to conduct genetic testing on the proband. A comprehensive set of 9,858,643,379 bases was derived from 65,289,029 paired-end read sequences during whole-exome sequencing. Among these, 97.87% of the reads exhibited a quality score (Q-score) exceeding 20, while 92.02% displayed a Q-score surpassing 30. Using the aforementioned analytical pipeline, more than 40,000 variants were identified. After eliminating polymorphisms in synonymous variants and intronic regions, the dataset retained 5,118 single nucleotide polymorphisms (SNPs) and 382 insertions/deletions (indels) for further analysis. Our focus was directed towards 50 genes most prominently associated with polydactyly on Genecards, which have been previously implicated in limb development during embryogenesis. Synonymous variants, variants located in the non-coding regions, variants with a minor allele frequency exceeding 0.01 in ExAC or the 1000 Genome Project, and variants with low potential for pathogenicity were excluded. Homozygous variants were also excluded from further analysis due to the apparent dominant inheritance pattern observed in the studied pedigree. Consequently, we identified a heterozygous substitution variant NM_000168.6:c.1384A>G (p.Lys462Glu) located in exon 10/15 of the *GLI3* gene. Sanger sequencing verification confirmed the inheritance of the genetic mutation by the proband from his father, who also manifested the disorder. Conversely, the unaffected mother did not exhibit this variant.

The *GLI3* c.1384A>G variant resulted in the substitution of lysine, a positively charged amino acid, with glutamic acid, a negatively charged residue. Multiple sequence alignment revealed a relative conservation of the amino acid position p.462K across various vertebrates (Figure 2). This conservation suggests that the sequence has been evolutionarily preserved due to its functional importance. Conserved sequences often indicate critical roles in the structure, function, or regulation of the protein, and changes in these regions may have significant biological implications.

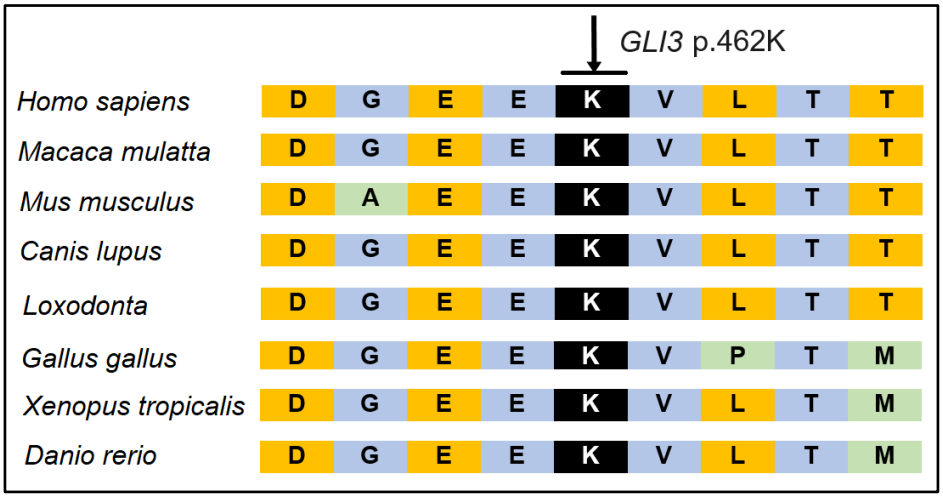

Figure 2. Conservation analysis of *GLI3* residues at amino acid position 462 across various species.

DISCUSSION

The understanding of the genetic basis of polydactyly has evolved with advancements in genetic research techniques such as whole exome sequencing, which allows for the identification of specific genetic alterations associated with the condition. This complexity underscores the importance of considering gene-gene interactions and the multifaceted nature of genetic contributions into account when studying and diagnosing polydactyly.

Polydactyly is linked to mutations occurring either in the coding sequence of specific genes or in the cis-regulatory regions governing gene expression. Mutations within the Hoxa or Hoxd clusters have been identified as causative factors for polydactyly. The synergistic interplay between *HOXD13* and *GLI3* has been implicated in synpolydactyly, characterized by additional and fused digits. Additionally, the Wnt signaling pathway and Notch contribute as supplementary signal transduction pathways (Lange *et al.*, 2014). Another illustrative example involves preaxial polydactyly, a congenital anomaly characterized by the occurrence of an extra thumb. This condition arises from ectopic production of the signaling molecule Sonic Hedgehog (SHH) during limb bud development. Mutations responsible for this anomaly result in aberrant expression of the long-distance, limb-specific cis-regulator for SHH, which is known as the Zone of Polarizing Activity Regulatory Sequence (ZRS) (Lettice *et al*., 2008). Nevertheless, significant gaps remain in our understanding of the molecular interaction network and signaling pathways associated with limb malformation and fetal development.

The *GLI3* gene encodes the GLI family zinc finger 3 protein, which is a transcription factor involved in the Hedgehog (Hh) signaling pathway. The GLI3 protein plays a crucial role in the regulation of embryonic development, particularly in the patterning of limbs and various other tissues. It acts downstream in the Hh signaling cascade, transducing signals from the Hh pathway, and controlling the expression of target genes involved in cell differentiation and tissue development (Sigafoos *et al*., 2021). Upon activation of the Hh pathway, the full-length isoform of GLI3 exerts its regulatory role on Hh-responsive genes by directly targeting the GLI1 promoter. Conversely, in the absence of Hh signaling, GLI3 undergoes phosphorylation events, resulting in partial degradation and the formation of the repressor isoform, GLI3-R, which functions to suppress Hh-mediated activities (Matissek, Elsawa, 2020). Beyond its canonical role in the Hh pathway, GLI3 is implicated in diverse biological processes, encompassing tissue development, immune cell differentiation, and neoplastic transformations.

The *GLI3* transcription factor plays a crucial role in the regulation of SHH signaling. Mutations in *GLI3* can lead to abnormal limb development, including polydactyly (Nguyen *et al.*, 2024). The SHH signaling pathway is essential for the patterning of digits during embryonic development. *GLI3* acts as a mediator in this pathway, and its protein product is involved in the transcriptional control of target genes. Mutations in the *GLI3* gene can cause dysregulation of SHH signaling, leading to the development of extra fingers or toes, characteristic of polydactyly (Al-Qattan *et al.*, 2017; Biesecker, 2006).

While the *GLI3* gene has been implicated in the etiology of polydactyly, syndactyly, and various congenital limb anomalies across multiple studies (Ngoc *et al*., 2020; Sczakiel *et al.*, 2021), it is noteworthy that the manifestation of these disorders is under the influence of a multitude of genes. In a recent investigation involving 78 Chinese children diagnosed with polydactyly, only a solitary case exhibited a mutation in the *GLI3* gene, while the remaining 77 did not carry mutations in either the *GLI3* or *SHH* genes (Rao *et al.*, 2018).

The variant detected in this study was located in the DNA-binding domain of GLI3 (Zinc finger domain). This domain enables GLI3 to bind specifically to DNA sequences, which is essential for the regulation of target gene expression. Once bound to DNA, GLI3 can regulate the transcription of target genes as an activator or repressor. Mutations in the zinc finger domain of GLI3 can disrupt its DNA-binding ability, leading to improper regulation of target genes. This disruption can result in limb malformation, such as a mutation in the *GLI3* zinc finger domain (*GLI3* c.1802A>G; p.His601Arg) that has been found in a large pedigree with polydactyly-syndactyly complex (Volodarky *et al.*, 2014); or another variant in this region (*GLI3* c.1482A>T; p.Gln494His) was detected in all affected members of a family associated with index finger polydactyly (Nguyen *et al.*, 2024).

Classifying recently discovered genes is currently necessary to aid medical professionals and researchers in understanding molecular etiology, the pathways involved, and rapid genetic diagnostics that could address medical challenges. Given that polydactyly is associated with hundreds of distinct syndromic illnesses, learning more about recently discovered pathways could help develop novel therapeutic interventions. Additionally, specific pathogenic variations in the causal gene may cause the observed phenotype, but phenotypic heterogeneity may be explained by a variety of polymorphisms in a complex gene-gene interaction network.

CONCLUSION

In conclusion, our study focused on a 6-year-old male proband with a distinctive unilateral polydactyly phenotype of the thumb in the right hand. Through whole-exome sequencing, we identified a heterozygous substitution variant (NM_000168.6: c.1384A>G; p.Lys462Glu) in the *GLI3* gene. Sanger sequencing confirmed the inheritance of this variant from the affected father. The amino acid change, substituting lysine with glutamic acid, was shown to be evolutionarily conserved, highlighting its potential functional significance. Our findings contribute to the understanding of the genetic basis of polydactyly and underscore the importance of *GLI3* for limb development. Further investigations are warranted to elucidate the broader implications of *GLI3* mutations in congenital limb abnormalities. This research extends beyond the laboratory, potentially influencing clinical practices, genetic counseling strategies, and ongoing efforts to advance our understanding of limb development disorders.

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CONFLICT OF INTEREST

The authors of this manuscript declare that they have no conflicts of interest to disclose. No financial or personal relationships with individuals or organizations could potentially bias the research or its outcomes.

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