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Crossed Polydactyly Type I Caused by a Point Mutation in the *GLI3* Gene in a Large Chinese Pedigree

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Polydactyly is one of the most common forms of congenital malformation in humans, and is displayed by 119 disorders. Crossed polydactyly (CP) is defined as the coexistence of preaxial and postaxial polydactyly with a difference in the axes of polydactyly between the hands and feet. In an effort to map the gene responsible for CP, we studied a seven-generation Chinese family of 56 individuals, 28 of whom were affected. A thorough search with highly informative polymorphic markers showed no recombination among the affected members with the markers on chromosome 7p15-q11.23, but no linkage with chromosomes 2q31, 7q36, 13q, and 19p. Mutation analysis showed a substitution mutation of 1927C→T in exon 12 of the *GLI3* gene, which is predicted to pretruncate the GLI3 protein. This mutation has variable phenotypes of polydactyly, indicating that other genetic factors also contribute to the diversity of polydactyly phenotypes. Our results increase the phenotypic spectrum caused by *GLI3* mutations and are important for the analysis and understanding of the etiology of these limb malformations. J. Clin. Lab. Anal. 20:133–138, 2006.

Key words: linkage; chromosome 7p15-q11.23; limb development; truncated protein

INTRODUCTION

Polydactyly is one of the most common forms of congenital malformation in humans and can occur either as a single malformation (nonsyndromic) or as part of multiple anomalies (syndromic). It is associated with diverse disorders, such as Greig cephalopolysyndactyly syndrome (GCPS) and Pallister-Hall syndrome (PHS) (1). According to the location of the extra finger or toe, polydactyly can be divided into two main types (pre- and postaxial polydactyly) and many subtypes. The crossed polydactyly type (CP) is defined as the presence of pre- and postaxial polydactyly with a difference in the axes of the polydactyly between the hands and feet. CP is divided into two types according to its clinical phenotypes: type I, in which postaxial polydactyly of the hand is combined with preaxial polydactyly of the feet; and type II, which is the opposite of type I (2).

In recent years, with the development in molecular techniques, the study of polydactyly has made great progress in mapping the chromosome loci involved and identifying gene mutations responsible for the phenotypes. Many loci for human polydactyly have been mapped, including synpolydactyly type II on chromosome 2q31 (3,4), triphalangeal thumb polydactyly on 7q36 (5), and postaxial polydactyly on 13q (6) and 19p (7). Furthermore, several genes have been identified as being involved in extra finger or toe formation, such as the transcription regulator gene *GLI3* (located on 7p15-q1.23) and *SHH* (located on 7q36). Human *GLI3* is homologous to the *Drosophila Cubitus interruptus* (*Ci*) and they have a similar function. *Ci* is a transcription regulator and an important component of the hedgehog signaling pathway for pattern formation of limbs and wings (8). Sonic hedgehog (SHH) is a morphogenetic signal produced by the polarizing region in the posterior limb bud. Ectopic anterior SHH expression induces digit

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duplications and is suspected to be a major cause of congenital limb malformations (9).

Polydactyly is genetically heterogeneous. Recent animal experiments suggest that *GLI3* and *SHH* mutations are caused by two distinct kinds of polydactyly (groups 1 and 2) (10). Group 1 has mirror-image ectopic limbs and is caused by the *SHH* mutation. The extra finger or toe is triphalangeal and characterized by mirror-image digit duplications. In contrast, group 2 is biphalangeal and characterized by a lack of mirror-image digit duplications, and is due to mutations in the *GLI3* gene. Different *GLI3* mutations result in various phenotypes that belong to group 2. To date, five autosomal-dominant polydactyly phenotypes have been confirmed to be associated with *GLI3* point mutation: GCPS (11), PHS (12), postaxial polydactyly type A (PAP-A) (13), postaxial polydactyly type A/B (PAP-A/B), and preaxial polydactyly type-IV (PPD-IV) (14). All phenotypes associated with *GLI3* mutation are termed "GLI3 morphopathies." It has been suggested that there is an association between the site of GLI3 protein truncation and the phenotypes caused by the *GLI3* mutation (12).

In this study we investigated the molecular basis of CP type I in an extended family of patients from southwestern China. To elucidate the etiology of polydactyly in this family, we selected D2S1384, D7S1179, D13S800, D13S154, D19S226, D19S929, D19S1165, D19S840, D19S558, D7S795, D7S521, D7S2454, and D7S1830 as polymorphic markers from the reportedly related chromosomes 2q31, 7q36, 13q 21-32, 19p13.1-13.2, and 7p15-q11.23. We mapped the responsible locus of the CP type I phenotype and analyzed the responsible gene mutation. We also addressed the hypothetical correlation between the phenotype and mutation site in the gene.

MATERIALS AND METHODS

Pedigree

This study was approved by the authorities of Yunnan Province and the Medical Ethics Committee of Yunnan University. All blood samples were collected with the informed consent of the subjects.

A large eight-generation kindred was found in Yunnan Province in southwestern China. This family had autosomal-dominant postaxial polydactyly of the hands combined with preaxial polydactyly of the feet with incomplete penetrance. Blood samples were collected from 56 individuals (28 affected patients and 28 normal relatives; the pedigree is shown in Fig. 1). The phenotype was highly variable within the family, and several hand/foot phenotypes are shown in Fig. 2. They include PAP-A/B in the hands and feet (Fig. 2a), duplication and syndactyly of the big toes (preaxial polydactyly of the feet, Fig. 2b), PAP-A in the bilateral hands and one of the feet (Fig. 2c), and fully bilateral PAP-A in the feet and hands (Fig. 2d). Most affected individuals had bilateral anomalies in their lower limbs and hands. Hand polydactyly was always observed combined with foot polydactyly. X-rays of selected individuals showed that additional fingers articulated with the fifth metacarpal, and the subjects had broad thumbs.

Polymorphisms and Linkage Analysis

Genomic DNA was purified from peripheral blood lymphocytes according to the standard SDS-proteinase-K

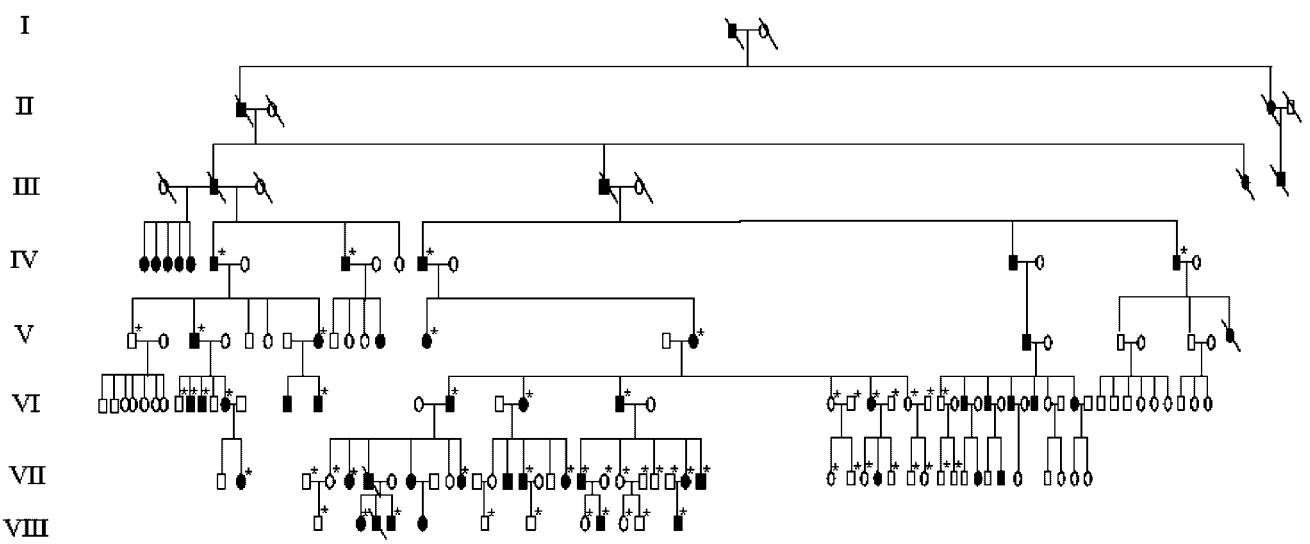

Fig. 1. Family tree of the CP pedigree. The participants in this study are labeled with a star.

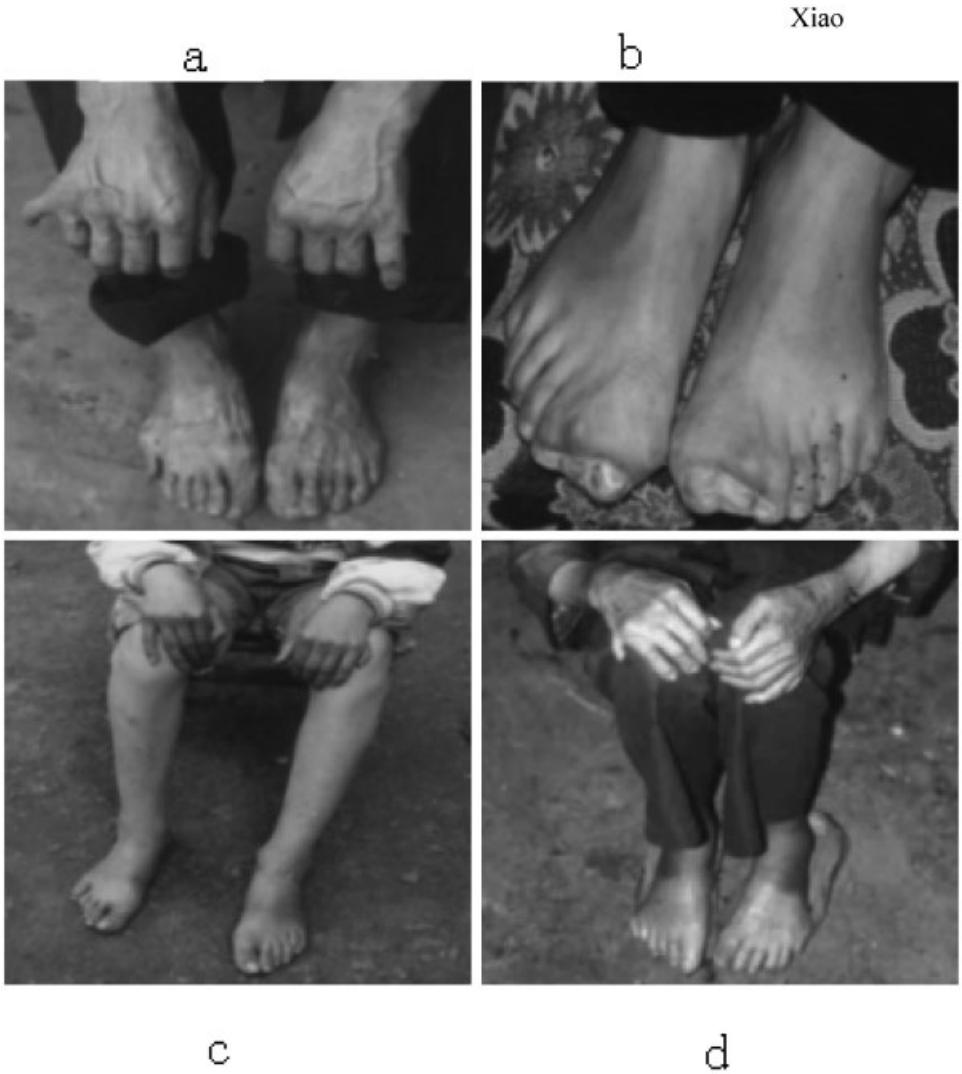

Fig. 2. **a–d:** Clinical photographs of four different affected individuals. See text for details.

and phenol/chloroform extraction method (15). DNA polymorphisms of short tandem repeats (STRs) were analyzed by PCR amplification. Polymorphic markers D2S1384 from chromosome 2q31, D7S1179 from 7q36; D13S800 and D13S154 from 13q 21-32; D19S226, D19S929, D19S1165, D19S840, and D19S558 from 19p13.1-13.2; and D7S795, D7S521, D7S2454, and D7S1830 from chromosome 7p15-q11.23 were selected for genotyping. The primer sequences were obtained from the Gene Database Bank (GDB). Single or duplex PCR amplifications were performed in a 25 μL reaction mixture containing 20 ng DNA, reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin), and 0.5 μL of each primer, 200 μM dNTPs, and 0.2 U Taq polymerase (Promega, Madison, WI). The PCR amplification of all markers was performed in a Perkin-Elmer 9600 at 95°C for 5 min followed by 15 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec, and then 15 cycles of 90°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec, and 72°C for 2 min. Electrophoretic separation of the amplified products was performed in a vertical denaturing (6 M urea) polyacrylamide gel (5%) using BRL SA32, with silver staining as described previously (16).

Linkage analysis of a single locus was performed using the LINKMAP program of LINKAGE version 5.2 (17).

Mutation Analysis

We used the oligonucleotide primers covering the 12th, 13th, and 14th exons of *GLI3* (18) to amplify the genomic DNA from the normal and affected individuals in the family. PCR amplifications were performed in a 25-μL reaction mixture containing 20 ng DNA, reaction buffer (10 mM Tris-HCl, pH 8.3,1.5 mM $MgCl_2$,

50 mM KCl, 0.001% gelatin), and 0.5 μL of each primer, 200 μM dNTPs, and 0.2 U Taq polymerase (Promega, Madison, WI). The PCR amplification was performed at 95°C for 5 min followed by 30 cycles of 95°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec. The PCR products were first run on agarose gels stained with ethidium bromide, purified with 75% alcohol, and then directly sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI Applied Biosystems, Foster City, CA). The sequences were aligned using the DNASTAR sequencer software.

RESULTS

The genotypes of different markers for every affected patient and normal relative were determined according to the product of amplification and then analyzed. The affected patients did not share the same genotype for markers from chromosomes 2q31, 7q36, 13q 21-32, and 19p13.1-13.2, which suggests that there was no linkage between these loci and the CP type I phenotype. Therefore, these four chromosomal regions can be excluded in the analysis of polydactyly in this pedigree. However, the affected patients shared the same genotype at the markers D7S795, D7S521, D7S2454, and D7S1830 located on chromosome 7p15-q11.23, and showed no recombination among the affected members. The suspected candidate gene in this region is *GLI3*. The 12th, 13th, and 14th exons of *GLI3*, in which most of mutations were reported, were sequenced.

The sequencing results for the *GLI3* gene showed that the affected members carried a substitution mutation in the heterozygosity form at nucleotide position 1927 (1927 C→T) in exon 12, which is not found in normal individuals. This results in a nonsense codon (CGA→TGA), which is predicted to cause a premature stop at amino acid position 643. The predicted truncation of the protein occurs between the zinc finger and domain 3 of *GLI3*. Sequence images of both sense and antisense directions are shown in Fig. 3.

DISCUSSION

Several loci were found to be associated with human polydactyly phenotypes, which demonstrates that polydactyly is genetically heterogeneous. In this pedigree the phenotype was highly variable within the family, and affected persons exhibited deformities such as fully bilateral PAP-A only in the lower limbs, PAP-A in the feet and hands, PAP-A/B in the hands, and PAP-A in the feet. One subject had both preaxial polydactyly and syndactyly in his big toes. Although it has been

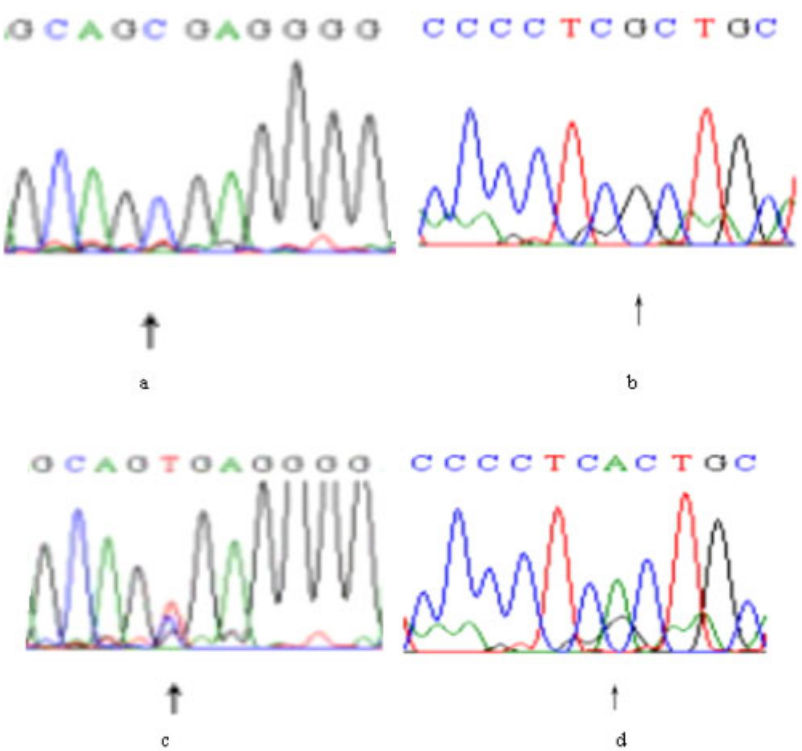

Fig. 3. A substitution mutation was identified in heterozygosity in exon 12 at nucleotide position 1927 (C→T). (**a**) Sense and (**b**) antisense sequences of a normal individual. (**c**) Sense and (**d**) antisense sequences of an affected mutation individual.

reported that preaxial polydactyly type IV (PPD-IV) is the same disorder as CP I (19), from this pedigree we can see that their phenotype differs significantly from the simple PPD-IV phenotype, that most of the affected members have an extra thumb or toe and the deformity of the extra thumb or toe is more severe in CP I. In this pedigree most of the polydactyly was located in the postaxial or central region of the hand. Because of the clear inherited pattern and the huge pedigree size, this family provided an excellent opportunity for us to explore the heterogeneous characteristics of this limb developmental abnormality.

According to our results, patients in this CP I pedigree showed no linkage between the phenotype and the loci 2q31, 7q36, 13q, and 19p, which are known to be involved with polydactyly. The markers located on chromosome 7p15-q11.23 such as D7S795 showed no recombination among the affected members, and the suspected candidate gene in this region is *GLI3*. In the exons we sequenced, a point mutation was identified in exon 12 in the affected persons. This was not found in the relatives with normal hand/feet development. This mutation is localized at position 1927, where a C changes to a T, and results in a stop codon formation. This stop codon is predicted to truncate the GLI3 protein at amino acid position 643 (Fig. 4). It occurs between the zinc finger and domain 3. The *GLI3* gene encodes a putative zinc finger transcriptional factor that is important in early vertebrate development and controls the number of fingers and toes in mammals (18) (Fig. 4a). It has been proposed that the GLI3 protein and the transcriptional factor dHAND are reciprocally antagonistic in the limb bud mesenchyma before the activation of SHH signaling (9). dHAND is required to activate SHH expression by the polarizing region cells (9). SHH signaling inhibits the processing of GLI3 and changes it to GLI3-83, which acts as a transcriptional suppressor. A truncated GLI3 protein (Fig. 4b) is the result of the mutated *GLI3* gene and its function mimics the GLI3-83, which functions as a transcriptional suppressor in early vertebrate development and does not control the number of fingers or toes in mammals (9). In this pedigree, the truncated GLI3 protein at amino acid position 643 acts as a transcriptional repressor in early vertebrate development and controls the number of fingers and toes, resulting in the CP1 polydactyly phenotype.

This same point mutation has also been reported in an Indian family (14), in which the phenotype differs from that in our study. In the Indian family, a total of 16 patients had the PAP-A/B phenotype, including four members with syndactyly of the second and third, and fifth and sixth toes, respectively. In the Chinese pedigree, only two of 28 members affected with polydactyly displayed syndactyly of the first and third toes. This shows that the same *GLI3* mutation has a variable phenotype. The fact that the same mutation has different manifestations may be attributed to a second modifier gene that harbors the mutation or a natural polymorphism and causes the different effects. A correlation was observed between the *GLI3* mutation genotype and the phenotypes of GCPS and PHS patients (20), in that the two syndromes are likely caused by mutations in the first third and the second third of the GLI3 protein, respectively. However, in our study a 1927 C→T mutation localizing in the first third of the protein led to a phenotype that was closer to the nonsyndromic type, which is not strictly consistent with the reported observation.

To date, five types of polydactyly (identified clinically) have been found to be caused by a point mutation in the *GLI3* gene. Our study identifies another dominantly inherited phenotype and thus expands the phenotypic spectrum caused by a *GLI3* gene mutation. We noticed that all six *GLI3*-associated polydactyly phenotypes in humans were non-mirror images and most of the extra fingers or toes were biphalangeal. In contrast, mirror-image digit triphalanges are associated with *SHH* (9,21). We suggest that *GLI3* is mainly involved in non-mirror-image biphalangeal polydactyly, and that mirror-image digit triphalanges are due to *SHH*. Furthermore, the classification of polydactyly by purely clinical means shows that this disease involves a complicated system. The phenotypic classification did not correlate with the molecular basis of the different types. Having identified the genetic mutation in each phenotype, we suggest that

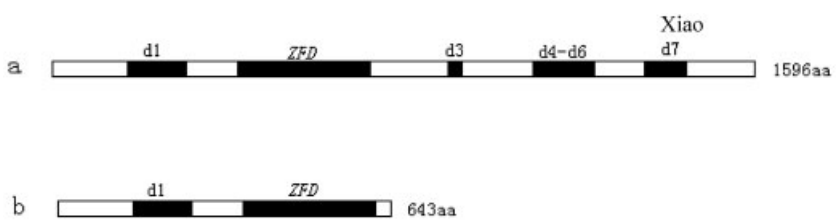

Fig. 4. **a:** Schematic drawing of normal human GLI3 protein. **b:** Schematic drawing of the truncated protein by the *GLI3* mutation in this pedigree. ZFD, zinc finger domain. d1–d7 are conserved domains of *GLI3* genes in different species.

the polydactyly in humans should be named by combining both the phenotype and the genetic mutation. For example, non-mirror-image biphalangeal polydactyly mainly caused by *GLI3* could be called "polydactyly of GLI3," and triphalageal polydactyly characterized by mirror-image digit duplications caused by *SHH* could be named "polydactyly of SHH." This would not only reduce the number of names used, it would also provide information about the genetic basis of the name.

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