Deciphering the mutational signature of congenital limb malformations

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INTRODUCTION

Congenital limb malformations (CLMs) affect 1 in 500 live births. However, the value of exome sequencing (ES) for CLM is lacking. The purpose of this study was to decipher the mutational signature of CLM on an exome level. We enrolled a cohort of 66 unrelated probands (including 47 families) with CLM requiring surgical correction. ES was performed for all patients and available parental samples. A definite molecular diagnosis was achieved in 21 out of 66 (32%) patients. We identified 19 pathogenic or likely pathogenic single-nucleotide variants and three copy number variants, of which 11 variants were novel. We identified four variants of uncertain significance. Additionally, we identified RPL9 and UBA2 as novel candidate genes for CLM. By comparing the detailed phenotypic features, we expand the phenotypic spectrum of diastrophic dysplasia and chromosome 6q terminal deletion syndrome. We also found that the diagnostic rate was significantly higher in patients with a family history of CLM (p = 0.012) or more than one limb affected (p = 0.034). Our study expands our understanding of the mutational and phenotypic spectrum of CLM and provides novel insights into the genetic basis of these syndromes.

Several types of genetic variants, including single-nucleotide variants (SNVs), small insertions and deletions (indels), and copy number variants (CNVs), have been implicated in CLM. The establishment of a genetic profile is essential to providing accurate genetic diagnosis and counseling, and to allow better understanding of disease prognosis. Despite the fact that there are >500 genes associated with CLM, many candidate genes remain under-recognized, and the majority of CLM cases cannot be diagnosed on a molecular level.

In order to investigate the molecular basis of CLM on an exome level, we performed exome sequencing (ES) in a cohort of 66 patients with CLM. In addition, to identify the mutational spectrum in known CLM-associated genes, we also searched for potentially novel CLM candidate genes and phenotypic expansions.

RESULTS

Clinical characteristics and diagnostic yield

We enrolled a total of 66 unrelated probands with CLM of Chinese Han ethnicity, including 41 males and 25 females. Forty-seven of them underwent family-based ES. Nineteen cases underwent one limb affected (p = 0.034). Our study expands our understanding of the mutational and phenotypic spectrum of CLM and provides novel insights into the genetic basis of these syndromes.

INTRODUCTION

Congenital limb malformation (CLM) represents a heterogeneous group of structural abnormalities of the limbs that originate from perturbations during limb development. CLM affects ~1 in 500 live births.1,2 While CLM is usually isolated, it can be present in conjunction with other congenital syndromes, such as Apert syndrome,3 Duane-radial ray syndrome,4 and Holt-Oram syndrome.5 About 20% of individuals with CLMs have at least one associated anomaly, and 10% of all congenital anomalies have upper limb involvement.6 Along with coexisting anomalies, CLM presents a significant psychological and clinical burden to affected individuals and their families.
proband-only ES. The mean age was 4.01 ± 0.58 years, with the majority (95%) being younger than 10 years old. Reduction anomalies were the most common anomaly in the cohort (25/66, 38%), followed by syndactyly (16/66, 24%), polydactyly (16/66, 24%), brachydactyly (6/66, 9%), and other unclassified anomalies (3/66, 5%). Overall, the characteristics of the cohort reflect a diverse distribution of surgically corrected CLM cases in clinical practice.

In total, 66 probands with CLM were analyzed. Of these, we established a molecular diagnosis in 21 patients (32%) (Figure 1). We identified 3 CNVs and 19 SNVs located in 10 genes (SALL4, FANCA, FGFR2, GLI3, HOXD13, UBA2, GJA1, and GDF5). All together, we identified 19 disease-causing SNVs (including 6 missense, 6 frameshift, 3 splice site, 1 nonsense, and 3 in-frame insertion) and 3 disease-causing CNVs (including deletion of 6q25.3, duplication of 7q36.3, and deletion of 16q24.3). Four variants of unknown significance (VOUS) were found in GLI3, FANCB, and SLC26A2. Among them, diagnostic yield was highly variable among different phenotypic groups (Table 1).

In our cohort, 32 cases (48%) have one limb affected and 34 cases (52%) have more than one limb affected. Thirty-four cases (52%) have syndromic anomalies, and 32 cases (48%) have isolated CLM. Fifty-one cases (77%) are sporadic, and 15 cases (23%) have a positive family history (Table 2). Diagnostic yield was higher in familial cases (60% versus 24%, p = 0.012) and cases with more than one limb affected (43% versus 16%, p = 0.034) (Table 2). Pathogenic and likely pathogenic variants and VOUS identified in the cohort are summarized in Table 3.

Pathogenic variants in known disease-causing genes
In total, previously reported causal variants in three genes were observed in six patients: FGFR2 (n = 4), GJA1 (n = 1), and HOXD13 (n = 1) (Table 3), accounting for 9% of all cases in the study (Figure 2). (1) Among these, we identified a heterozygous FGFR2 variant, c.758C > G (p.Pro253Arg), in patients DISCO-JST22, DISCO-JST39, and DISCO-JST51. Notably, four patients presented with features characteristic of Apert syndrome, such as craniosynostosis, midface hypoplasia, and syndactyly of the hands and feet. (2) We also found a de novo heterozygous FGFR2 variant, c.755C > G(Ser252Trp), in patient DISCO-JST48, who presented typical features of Apert syndrome. These two FGFR2 variants have been reported in multiple patients with Apert syndrome.3,4 Interestingly, an in-frame insertion variant of RYR1, c.12788_12793dup (Glu4263_Gly4264dup), was also observed in patient DISCO-JST48, as well as in his father and sister. Patient DISCO-JST48 had a history of malignant hyperthermia during surgery 2 years prior to this study. This c.12788_12793dup (Glu4263_Gly4264dup) has not been previously reported in the literature but occurred four times as a heterozygous mutation in the Genome Aggregation Database (gnomAD) database. However, patient DISCO-JST48’s father and sister had no history of anesthesia and no signs of malignant hyperthermia. Thus, this variant is considered a VOUS. (3) A heterozygous GJA1 c.119C > T (p.Ala40Val) variant was found in patient DISCO-JST22, who presented with syndactyly and dental dysplasia. This variant has previously been found in one Japanese patient with oculodentodigital dysplasia.5 (4) Patient DISCO-JST33, who presented with central synpolydactyly, was found to carry a HOXD13 c.917G > A (p.Arg306Gln) variant, which has previously been identified in multiple patients with synpolydactyly.6,11

Novel variants in known disease-causing genes
In order to expand our understanding of the mutational landscape of CLM, we focused on novel variants that have not been previously implicated in CLM. We identified nine novel truncating variants in five genes: SALL4 (n = 4), GLI3 (n = 2), SLC26A4 (n = 1), FANCA (n = 1), and CREBBP (n = 1) (Figure 2). These variants include six frameshift variants, two splice variants, and one nonsense variant. Pedigree, clinical pictures, and results of Sanger sequencing of these
families are presented in Figure 3. Notably, one novel missense variant and one novel in-frame insertion variant were observed in two patients (Table 3).

A heterozygous \( GDF5 \) c.932T > C (p.Leu311Pro) variant was identified in patient DISCO-JST17 and her mother, both of whom presented with brachydactyly type C (BDC) (Figure 3C). Two additional family members of patient DISCO-JST17’s mother also presented with similar phenotypes, but genetic tests were not performed. This variant is absent from the gnomAD database and is predicted to be deleterious by multiple bioinformatic tools (CADD = 19.42, SIFT = 0, gerp++ = 4.75 and PolyPhen2 HDIV = 0.997). Thus, we considered \( GDF5 \) c.932T > C (p.Leu311Pro) to be a likely pathogenic variant.

Patient DISCO-JST57 had syndactyly of 3rd and 4th fingers of the right hand (Figure 3H). In addition, her mother and five family members within her mother’s family presented with bilateral 2nd toe clinodactyly. ES of the proband and her parents identified an in-frame insertion variant, c.183_203dup (p.Ala65_Ala71dup), in \( HOXD13 \), which is absent from the gnomAD database. This variant causes the number of residues in a polyalanine tract of \( HOXD13 \) to increase from 15 to 22. Although this cDNA change was not reported previously, different cDNA changes leading to the polyalanine tract increasing from 15 to 22 residues were observed in several families with syndactyly or clinodactyly.12

### Phenotypic spectrum expansion in known CLM genes

In our cohort, phenotypic expansion was associated with one gene (\( SLC26A2 \)) and one CNV (6q25.3-6q27). Patient DISCO-JST8 presented with limb shortening, intrauterine growth retardation, language retardation, decreased testicular size, and normal skull size. ES found that DISCO-JST8 carried two \( SLC26A2 \) variants (Figure 3B). The c.1512G > A (p.Met504Ile) was inherited from his father, and the c.136_137insTT (p.Asp46Valfs*44) was not inherited from his father (mother not tested). Genotype and phenotype of patient DISCO-JST8 strongly suggest the diagnosis of diastrophic dysplasia (DTD), although we are unable to determine whether the variants occur in trans or in cis because of the unavailability

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**Table 1. Diagnostic yield among different phenotypic groups**

| Phenotypic Group                          | Total (no.) | Diagnosed (no.) | Undiagnosed (no.) | Diagnostic yield (%) | Genes (no. of patients) |
|------------------------------------------|-------------|-----------------|--------------------|----------------------|-------------------------|
| Reduction anomaly                         | 25          | 7               | 18                 | 28                   | SALL4 (4)               |
| Duane-radial ray syndrome                 | 7           | 4               | 3                  | 57                   |                         |
| SHFM                                     | 3           | 0               | 3                  | 0                    |                         |
| Holt-Oram syndrome                        | 2           | 0               | 0                  | 0                    |                         |
| Longitudinal reduction                    | 2           | 0               | 2                  | 0                    |                         |
| Epiphyseal dysplasia                      | 1           | 1               | 0                  | 100                  |                         |
| Diamond-Blackfan anemia                   | 1           | 1               | 0                  | 100                  |                         |
| Fanconi anemia                            | 1           | 1               | 0                  | 100                  |                         |
| Nager syndrome                            | 1           | 0               | 1                  | 0                    |                         |
| Unclassified radial reduction             | 7           | 0               | 7                  | 0                    |                         |
| Syndactyly                               | 16          | 8               | 8                  | 50                   |                         |
| Apert syndrome                            | 4           | 4               | 0                  | 100                  |                         |
| Unclassified                             | 12          | 4               | 8                  | 33                   | GLI3(1); HOXD13(1); GJA1(1); UBA2(1) |
| Polydactyly                               | 16          | 6               | 10                 | 38                   |                         |
| Preaxial polydactyly                      | 10          | 2               | 8                  | 20                   |                         |
| Postaxial polydactyly                     | 2           | 0               | 0                  | 0                    |                         |
| Syndactyly                               | 2           | 2               | 0                  | 100                  | HOXD13(2)               |
| Greig cephalopolysyndacty syndrome        | 1           | 1               | 0                  | 100                  |                         |
| Rubinstein-Taybi syndrome                 | 1           | 1               | 0                  | 100                  |                         |
| Brachydactyly                             | 6           | 1               | 5                  | 17                   |                         |
| Poland anomaly                            | 5           | 0               | 5                  | 0                    |                         |
| Brachydactyly type C                      | 1           | 1               | 0                  | 100                  | GDF5(1)                 |
| Others                                   | 3           | 0               | 2                  | 33                   |                         |
| Constraint band syndrome                  | 2           | 0               | 2                  | 0                    |                         |
| Madelung deformity                        | 1           | 0               | 1                  | 0                    |                         |
| Total                                     | 66          | 21              | 45                 | 32                   |                         |

SHFM, split hand/foot malformation; No., number.
the biological association between RPL9 and other ribosomal protein family, such as RPS10 and RPS26, have been associated with DBA, whereas language retardation and testicle hypoplasia is not associated with DTD.

Patient DISCO-JST24 presented with congenital heart disease, developmental delay, structural brain anomalies, craniofacial anomalies, constipation, preaxial polydactyly, and elevated blood ketone bodies and dicarboxylic acid levels have not been previously reported as manifestations of chromosome 6q deletion.14 Although terminal deletion of chromosome 6q (6q25.3-6q27 in this case) is associated with variable phenotype specificity, the patient’s other symptoms of constipation, preaxial polydactyly, and elevated blood ketone bodies and dicarboxylic acid levels have not been previously reported as manifestations of chromosome 6q deletion.14

| Table 2. Diagnostic yield comparison in patients with different clinical characteristics |
|---------------------------------|-----------------|-----------------|-----------------|
| Total (no.) | Diagnosed (no.) | Undiagnosed (no.) | Diagnostic yield (%) |
| Family history |
| Yes | 15 | 9 | 6 | 60 |
| No | 51 | 12 | 39 | 24 |
| p value | 0.012 |
| No. of affected limb(s) |
| 1 | 32 | 5 | 27 | 16 |
| ≥ 2 | 34 | 16 | 21 | 43 |
| p value | 0.034 |
| Systemic anomalies |
| Syndromic | 34 | 13 | 21 | 38 |
| Isolated | 32 | 8 | 24 | 25 |
| p value | 0.297 |

No., number.

Identification of novel candidate genes for CLM

In order to uncover novel genes potentially contributing to CLM, we searched for new candidate genes that have not been reported in CLM. As a result, we identified a de novo heterozygous start-loss variant, c.-2+2T > A, in RPL9 from patient DISCO-JST24, a 2-year-old female born to healthy parents (Figure 3J). This variant was absent from the gnomAD database. The patient presented with bilateral thumb hypoplasia, cleft palate, dental dysplasia, microgastria, high scapula, and mild anemia (Figure 3J). She is clinically diagnosed with Diamond-Blackfan anemia (DBA), which is an inherited bone marrow failure disorder, along with a number of other systemic anomalies, including thumb hypoplasia and craniofacial defects. Although other members of the ribosomal protein family, such as RPS10 and RPS26, have been associated with DBA,13 RPL9, which encodes ribosomal protein L9, is not an established disease gene. Considering the biological association between RPL9 and other ribosomal proteins, the truncating variant observed in patient DISCO-JST24 provides evidence supporting a causal role of RPL9 in DBA.

Patient DISCO-JST19 was a 3-year-old male born with an aplasia cutis lesion at vertex, which is ~5 cm in diameter (Figure 3K). His weight and height at 3 years old were 12 kg (<3 SD) and 86 cm (<3 SD), respectively. He had cutaneous syndactyly of 3rd and 4th fingers in the right hand (Figure 3K), which was corrected by a syndactyly release surgery. After the surgery, he developed contractures in these two fingers. Other anomalies include hypoplasia and large and low-set ears. He had no Duane anomaly and strabismus. His developmental milestone was normal. Review of his family members revealed no history of aplasia cutis congenita (ACC), digital anomalies, or other syndromes. ES identified a de novo heterozygous variant, c.811A > T(p.Lys271Ter), in the UBA2 gene (Figure 3K). This is predicted to lead to a premature stop codon and is absent from the gnomAD database.

DISCUSSION

By applying ES in a cohort of 66 CLM patients, we found that 21/66 (32%) families within our cohort could be molecularly diagnosed. We identified six known and 11 novel pathogenic/likely pathogenic variants in known CLM-associated genes and revealed two potential novel candidate genes. Additionally, we observed phenotypic expansions for one known CLM-associated gene and CNV.

Limb development is a multi-stage process, which is orchestrated by complex interactions between different signaling pathways. Studies have found that multiple biological pathways are critically involved in the development of limbs.16,17 Any perturbation of these pathways could result in CLM. For example, mutations in the WNT pathways have been shown to lead to Robinow syndrome, which is characterized by brachydactyly.18,19 Mutations in Cohesin proteins could result in Cornelia de Lange syndrome, which presents clinodactyly and oligodactyly, in the context of limb anomaly.20 Besides genes with sufficient knowledge to be classified in certain biological pathways, many CLM-related genes are still not well studied.

With the advent of high-throughput sequencing technology, >500 CLM-related genes have been identified.21 The genetic bases of different kinds of CLM differ from each other but also share some similarities. The majority of polydactyly is caused by mutations in Gli3, which could also lead to syndactyly.22 The HOXD13 mutations can either present clinodactyly or syndactyly, even in the same pedigree.23 In this study, we identified 19 causal SNVs and three causal CNVs in 66 CLM patients, which reflect the diversity of CLM causative genes. Thus, we suggest using ES as first-line strategy to identify the genetic basis of CLM.

A previous study used a targeted next-generation sequencing (NGS) strategy encompassing 52 CLM-associated genes along with their regulatory regions in a cohort of 352 CLM patients. They found that 35.2% patients could be molecularly diagnosed, a rate similar to that in our study. However, we identified a different mutational architecture: only 6 among 15 genes identified in our study were included.
Table 3. Summary of pathogenic and likely pathogenic variants identified in the cohort

**Known Genes**

| CaseID      | Limb presentations | Systemic features | Family history | No. of affected limbs | Locus | Zygosity | cDNA change | Protein change | ACMG grade | Reference (PMID)     |
|-------------|--------------------|-------------------|----------------|-----------------------|-------|----------|-------------|---------------|------------|-----------------|
| DISCO-JST1  | reduction anomaly  | Y                 | Y              | B-H                   | SALL4 | Het      | c.1823del   | p.Asn608Thrfs*2 | P          | this study      |
| DISCO-JST8  | reduction anomaly  | Y                 | N              | B-H, B-F              | SLC26A2 | NA*     | c.136_137insTT | p.Asp46Valfs*44 | P          | this study      |
| DISCO-JST13 | syndactyly         | Y                 | N              | B-H, B-F              | FGFR2 | Het      | c.758C > G  | p.Pro253Arg    | P          | 7719434         |
| DISCO-JST17 | brachydactyly      | N                 | Y              | B-H                   | GDF5  | Het      | c.932T > C  | p.Leu311Pro    | LP         | this study      |
| DISCO-JST22 | syndactyly         | Y                 | Y              | B-H, L-F              | GJA1  | Het      | c.119C > T  | p.Ala40Val     | P          | 25327171 and   |
| DISCO-JST28 | polydactyly        | Y                 | N              | B-H, B-F              | GLI3  | Het      | c.1474del   | p.Asp492Thrfs*10| P          | 15879313        |
| DISCO-JST29 | reduction anomaly  | Y                 | N              | B-H                   | FANCA | Hemi     | c.3537+2T > C| p.?           | P          | this study      |
| DISCO-JST33 | synpolydactyly     | N                 | Y              | R-H                   | HOXD13 | Het      | c.917G > A  | p.Arg306Gln    | P          | 22374128 and   |
| DISCO-JST39 | syndactyly         | Y                 | N              | B-H, B-F              | FGFR2 | Het      | c.758C > G  | p.Pro253Arg    | P          | 24789103        |
| DISCO-JST41 | reduction anomaly  | Y                 | Y              | B-H                   | SALL4 | Het      | c.595del    | p.Asp199Metfs*41| P          | this study      |
| DISCO-JST47 | reduction anomaly  | Y                 | Y              | B-H                   | SALL4 | Het      | c.2462-1G > T| p.?           | P          | this study      |
| DISCO-JST48 | syndactyly         | Y                 | N              | B-H, B-F              | FGFR2; RYR1 | Het; Het | c.755C > Gc.12788_12793dup | p.Ser252Trp; p.Glu4263_Gly4264dup; P; VOUS | this study |
| DISCO-JST51 | syndactyly         | Y                 | N              | B-H, B-F              | FGFR2 | Het      | c.758C > G  | p.Pro253Arg    | P          | 7719434         |
| DISCO-JST57 | syndactyly         | N                 | Y              | R-H                   | HOXD13 | Het      | c.183_203dup | p.Ala65_Ala71dup| P          | this study      |
| DISCO-JST70 | syndactyly         | N                 | N              | B-H, B-F              | GLI3  | Het      | c.480del    | p.Ala161Profs*55| P          | this study      |
| DISCO-JST72 | reduction anomaly  | Y                 | Y              | R-H                   | SALL4 | Het      | c.1746_1747 delinsTGTGGG | p.Lys582Asnfs*17| P          | this study      |
| DISCO-JST74 | broad thumbs       | Y                 | N              | R-H                   | CREBBP | Het      | c.4471C > T  | p.Gln1491*     | P          | this study      |

**Novel candidate genes**

| CaseID      | Limb presentations | Systemic features | Family history | Affected site | Locus | Zygosity | cDNA change | Protein change | gnomAD MAF |
|-------------|--------------------|-------------------|----------------|---------------|-------|----------|-------------|---------------|------------|
| DISCO-JST24 | reduction anomaly  | Y                 | N              | B-H           | RPL9  | Het      | c.-2+2T > A | p.?           | 0          |
| DISCO-JST19 | syndactyly         | N                 | N              | R-H           | UBA2  | Het      | c.811A > T  | p.Lys271Ter   | 0          |

(Continued on next page)
in the reported panel. Indeed, there is epidemiological variation of CLM among different regions and demographics,2,21,23 which might explain the difference of mutational architecture. Using a targeted NGS strategy is time-saving in clinical practice. However, >500 genes are associated with CLM, and the genetic cause of CLM remains largely elusive. Using an exome-wide or genome-wide strategy has the potential to establish an unbiased genetic landscape and uncover novel candidate genes.

In our cohort, we found that the diagnostic yield was higher in familial cases and cases with more than one limb affected (Table 2). Previous studies have drawn similar conclusions regarding the existence of family history and systemic abnormalities and the ability of these factors to affect diagnostic yield.7 In this study, we describe for the first time how the number of affected limbs can influence the diagnostic yield. This might be helpful for clinical geneticists to provide precise management of patients with CLM.

We identified a start-lost variant in RPL9, which is not a well-established disease gene. Previously, this RPL9 variant has only been found in one patient with DBA.15 Since the phenotypical manifestation of our patient is highly similar to DBA, and DBA is caused by variants in the ribosomal protein family with only a few exceptions, we concluded that RPL9 may represent a potential novel disease gene candidate for DBA.24 Additionally, the variant leads to start-loss of RPL9 protein, and the probability of loss-of-function intolerance (pLI) score of RPL9 is 0.96. Nevertheless, further studies are still needed to confirm the relationship between RPL9 and DBA.

We identified a de novo nonsense UBA2 variant in a patient with ACC, intrauterine and postnatal growth retardation, syndactyly, atrial septal defect, hypospadias, and large and low-set ears. These phenotypes are also typical features in 19q13.11 deletion syndrome.25 Among patients with 19q13.11 deletion, most of them had ACC and most male patients had underdelivered genitalia, as observed in our case. After analyzing the correlation between clinical features and deleted regions, Melo et al.26 hypothesized that haploinsufficiency of UBA2 gene is responsible for ACC. This hypothesis was further supported by the identification of UBA2 variants in ACC patients. Marble et al.27 reported a patient with ACC, Duane anomaly, and hip dysplasia, who carried a de novo missense variant c.71G > T (p.Gly24Val) in UBA2. Wang et al.28 reported a male child and his mother both affected with ACC and found to have a co-segregating truncating variant, c.327delT(p.Phe109Leufs*5), while, in a recent study about split-hand/foot malformation, Yamoto et al.25 identified a UBA2 frameshift variant, c.1324dupT p.(Tyr442Leufs*17), in a patient affected with split hand/foot malformation but not ACC. Our findings strengthen the hypothesis that UBA2 is a novel candidate gene for Mendelian ACC and provide novel insights in relating UBA2 with syndactyly.

### Table 3. Continued

| Structural variants | Case ID | Limb presentations | Systemic features | Family history | Affected site | Locus | Zygosity | Dosage | Lineage | Protein change | gnomAD MAF |
|---------------------|---------|-------------------|------------------|---------------|---------------|-------|---------|--------|---------|---------------|------------|
| DISCO-JST35         | syndactyly | N | N | L-H | GLI3 | Het | N | Y | "-" | p.Ala35Thr | 0 |
| DISCO-JST55         | syndactyly | N | N | R-H | GLI3 | Het | N | Y | "-" | p.Ala1139Ser | 4.01E-06 |
| DISCO-JST8          | reduction anomaly | Y | N | R-H | SLC26A2 | NAa | c.1512G > A | p.Met504Ile | 2.76E-4 |

Y, yes or present; N, not present; No, number; H, hand; F, foot; Het, heterozygous; B, bilateral; R, right; L, left; Hom, homozygous; P, pathogenic; LP, likely pathogenic; VOUS, variant of unknown significance; MAF, minor allele frequency; NA, not available.

aDue to lack of maternal sample, the zygosity cannot be determined.
All together, we performed ES on a cohort of 66 Chinese patients with CLM. Our findings of previously reported variants confirmed the pathogenicity of those variants and the validity of our protocol. We also found several novel variants that expand the mutational architecture. Finally, we identified RPL9 and UBA2 as possible novel candidate genes for CLM.

MATERIALS AND METHODS

Cohort recruitment and sample preparation

In this study, we enrolled a total of 66 unrelated probands with surgical corrective CLM admitted to Beijing Jishuitan Hospital, China from April 2018 to September 2019 as a part of the Deciphering disorders Involving Scoliosis and Comorbidities study (http://www.discostudy.org/), as well as 47 pairs of patient parents. Medical records, X-rays, clinical images, and blood samples were collected for each patient. Limb and systemic anomalies of these patients were evaluated by experienced hand surgeons and pediatricians. Patients were classified into five phenotypic groups. The reduction anomaly group included patients with Duane-radial ray syndrome, split hand/foot malformation, Holt-Oram syndrome, multiple epiphyseal dysplasia, DBA, Fanconi anemia, and unclassified radial anomalies. The syndactyly group consisted of patients with Apert syndrome and isolated syndactyly (unclassified). The polydactyly group included patients with preaxial polydactyly, postaxial polydactyly, synpolydactyly, Greig cephalopolysyndactyly syndrome (GCPS), and Rubinstein-Taybi syndrome (RTS). The brachydactyly group included patients with Poland anomaly and BDC. Several other conditions were also screened in our cohort, including constraint band syndrome and Madelung deformity. After collection of blood samples, genomic DNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer’s protocol. This study was approved by the ethics committee of Beijing Jishuitan Hospital. Informed consent was obtained from each patient or their parents.

Exome sequencing and data processing

In brief, DNA samples were prepared into Illumina paired-end libraries and underwent whole-exome capture with the Agilent V5, followed by sequencing on the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA). In-house-developed Peking Union Medical College Hospital Pipeline (PUMP) was used for variant calling and annotation.29,30

An in-house control dataset of 100 exomes was incorporated for CNV analysis. Coverage information was computed from BAM files with GATK. Then, unqualified targets with fewer than 50% samples covered or with a mean coverage under 10× were filtered out. After quality control, read depths for each sample were analyzed and a Z score was calculated. CNVs were called according to the Z score.

ES data interpretation

Rare variants with minor allele frequencies < 0.01 in 1000 Genomes (October 2013), gnomAD (https://gnomad.broadinstitute.org), the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org), and the in-house database of Deciphering Disorders Involving Scoliosis and Comorbidities (DISCO, http://discostudy.org/, >2,000 exomes) were extracted. First, we examined disease-causing variants in previously reported candidate genes and CNVs. Variant classification was conducted according to American College of Medical Genetics and Genomics (ACMG) guidelines.31 For patients highly suspected of a specific syndrome but without a previously identified pathogenic/likely pathogenic variant, we prioritized potential new variant(s) in the disease-associated gene(s). First, truncating variants in genes predicted to be intolerant to loss-of-function changes according to the gnomAD (pLI score ≥ 0.9) were identified as susceptibility variants. Variants that are predicted with a CADD score of >15 were presumed damaging. Candidacy of rare and damaging variants was further evaluated by their known gene function, animal models, and association with known CLM genes.

Validation of candidate variants

Variant-encoding amplicons were amplified by PCR from genomic DNA obtained from subjects, purified with an Axygen AP-GX-50 kit (lot no. 05915KE1), and sequenced by Sanger sequencing on an ABI3730XL instrument.
ACKNOWLEDGMENTS
We thank the families who participated in this research. This research was funded in part by the National Key Research and Development Program of China (2016YFC0901500); Center for Rare Diseases Research, Chinese Academy of Medical Sciences, Beijing, China (2016ZX310174-4); Beijing JST Research Funding (ZR-201907 and 2019-Y03); Beijing Jishuitan Hospital Nova Program (XKKX2018); National Natural Science Foundation of China (81822030, 82022391, 81672123, 81972037, 81772299, 81930068, 81772301, and 81972132); Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (no. 2019PT320025); Beijing Natural Science Foundation (JQ20032 and 7191007); Tsinghua University-Peking Union Medical College Hospital Initiative Scientific Research Program; CAMS Initiative Fund for Medical Sciences (2016-I2M-3-003, 2016-I2M-2-006, and 2017-I2M-2-001); and the National Students Innovation and Entrepreneurship Training Program (2019zlgc0609). This work was performed in Beijing, China.

AUTHOR CONTRIBUTIONS
Conceptualization: W.T., Z.W., T.J.Z., and N.W.; cohort enrollment: L.S., Y.G., M.L., Z.Z., Z.Y., and Y.H.; funding acquisition: N.W., Z.W., W.T., and T.J.Z.; experiments: Z.C., L.W., Y.Z., Y.L., and Y.H.; genetic data analysis: Y.H., L.S., S.Z., Z.Y., and N.W.; bioinformatic analysis: Z.Y., S.Z., and Z.C.; writing – review & editing: T.J.Z., G.Q., J.Z., and Z.W.; data interpretation: Z.W., T.J.Z., and N.W.; writing – original draft: Y.H., L.S., S.Z., J.Z., Z.Y., Y.G., X.D., W.T., and N.W.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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