Whole-exome sequencing study identifies two novel rare variations associated with congenital talipes equinovarus

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Abstract. Congenital talipes equinovarus (CTEV) is a common birth defect with an unclear genetic pathogenesis that results from both genetic and environmental factors. The present study aimed to identify novel variants in patients with CTEV using whole-exome sequencing (WES) and to investigate the genetic factors responsible for the development of CTEV. A cohort of nine neonates/infants with suspected CTEV was recruited. Subsequently, sequential tests, including chromosome karyotyping and WES, were performed for each of the participants. Familial validation was performed using Sanger sequencing and low-coverage copy-number variation (cnV) sequencing. A novel cnV containing the mediator complex subunit 13l gene at 12q24.21-q24.23 was detected by WES and further investigated by cnVseq. Additionally, a novel de novo missense variation, transforming growth factor-β receptor 2: c.1280T>C, was identified by WES and further investigated by Sanger sequencing. The two identified variations were hypothesized to be causative genetic factors for the development of CTEV in the two cases the variations were identified in. In the present study, two pathogenic variations (one cnV and one single-base variation) were detected in two Chinese families with CTEV. The results of the present study may aid in investigating the molecular basis of CTEV; however, further investigation is required.

Introduction

Congenital talipes equinovarus (CTEV), also known as clubfoot, is one of the most common inborn musculoskeletal abnormalities, with a worldwide incidence of 1 in 1,000 livebirths (1). CTEV is identified by four clinical foot characteristics: Forefoot adduction, hindfoot varus, midfoot cavus and hindfoot equinus (2). A total of ~80% of CTEV cases are idiopathic, namely ICTEV (3), with the remaining 20% being characterized as secondary or syndromic CTEV (4). ICTEV affects males more than females (5), with a male-to-female ratio of 2:1 across different ethnic groups (1,6).

Although a number of studies on familial characteristics, especially twins, have strongly suggested that genetic factors play an important role in the pathogenesis of ICTEV (4,7-9), the particular locality of the etiology is still unclear compared with syndromic CTEV (5).

Previous studies on the association between ICTEV and genetic factors have identified several causative gene families and genes, including Hox (10,11), caspase (2,11), paired like homeodomain 1 (7-9,12), T-box transcription factors and GlI family zinc finger 3 (10,13) genes. However, a major candidate gene remains to be identified (5). Previous studies have focused on the interaction between genetic and environmental factors, displaying the multifactorial identity of the disease (4,5,14,15). At present, multifactorial identity remains the most validated theory (5). Additionally, numerous other phenotypes of syndromic clubfoot in newborns are not recognizable and can be easily misdiagnosed as ICTEV (16,17). Accordingly, these patients require the development of improved molecular methods for accurate differential diagnosis.

In the present study, whole-exome sequencing (WES) was used to investigate nine neonates/infants with CTEV, among which seven displayed isolated CTEV and two displayed CTEV combined with other abnormalities.

Materials and methods

Subjects. The present study was approved by the Ethics Committee of Shijiazhuang Obstetrics and Gynecology Hospital (approval no. 20180015) and written informed consent was obtained from all patients. Whole-exome sequencing (WES) was performed for all participants.

Key words: congenital talipes equinovarus, whole-exome sequencing, mediator complex subunit 13L, transforming growth factor-β receptor 2
consent was obtained from the parents of all patients. Patients with suspected CTEV, identified by routine clinical and ultrasound diagnostic criteria (2) without clear genetic diagnosis, were recruited for the present study. Patients with isolated (both bilateral and unilateral) and combined CTEV were included in the present study. The exclusion criteria were as follows: i) Clear prenatal genetic diagnosis; ii) mechanical injury during labor. A total of nine neonates or infants with CTEV were recruited from the Center of Prenatal Diagnosis in Shijiazhuang Obstetrics and Gynecology Hospital and the Department of Pediatric Orthopedic in The Third Hospital of Hebei Medical University between January 2016 and December 2018. Patient characteristics are shown in Table I. Peripheral blood (5 ml) was collected from each subject for genetic testing.

Chromosome karyotyping. Conventional chromosome karyotyping by G-banding was performed on the peripheral blood samples to detect overall chromosomal anomalies, as previously described (18). The karyotype results were analyzed in accordance with the International System for Human Cytogenomic Nomenclature (2016 edition) (19).

DNA extraction. Total genomic DNA (1 µg) was extracted from 200 µl peripheral blood using the DNA Blood Midi/Mini kit (Qiagen GmbH), according to the manufacturer's protocol.

WES analysis. DNA library construction, quality testing and WES experiments were performed as previously described (18); however, the 'proband only' analysis strategy was adopted for the identification of causative variants. WES analysis was performed using the Novaseq6000 platform (Illumina). Sequencing reads were mapped to the human reference genome (hg19/GRC37) and underwent standard quality control screening. The Verita Trekker® Variants Detection system (Berry Genomics, Inc.) was used to identify single-nucleotide polymorphisms, insertion and deletions, copy number variants (CNVs), mitochondrial gene variants and runs of homozygosity. Subsequently, the Enliven® Variants Annotation Interpretation (Berry Genomics, Inc.) system was used to fulfill the annotation and interpretation progress referring to multiple databases. The overall workflow is presented in Fig. 1. After the variation filtering process, several disease-associated variants were identified and selected for familial validation using Sanger or CNV sequencing as previously described (20).

To identify the novel missense variation, homologous analysis among species was performed using the NCBI blast online software (blast.ncbi.nlm.nih.gov/Blast.cgi; 2019/Oct/29). In silico analysis was performed using Sorting Intolerant from Tolerant (SIFT; sift.bii.a-star.edu.sg; 2019/Oct/29) and Polymorphism Phenotyping (PolyPhen; version 2; genetics.bwh.harvard.edu/pph2) software to calculate the respective pathogenicity indices. Biophysical analysis was conducted using Modeller software (version 9.21; saiblab.org/modeller).

Results

Clinical data. The main clinical data are presented in Table I. All the subjects were patients with ICTEV with no family history of the disease (Table I). The parents had no adverse habits, for example, smoking. Representative clinical and ultrasonic illustrations are presented in Fig. 2.

Genetic analysis. The karyotyping results were normal for each of the subjects. However, two potentially causative variations were identified by WES and were further investigated by familial validation. The two potential causative variations identified were: seq[GRC37]del(12)(q24.21-q24.23)g.116399065-120555197 encompassing the mediator complex subunit 13L (Med13L) gene in case 1 and transforming growth factor-β receptor 2 (TGFB2):NM_001024847.2:exon5:c.1280T>c:p.l427P in case 3 (Fig. 3 A and B). Several suspected variations were detected in other cases, but did not match the inheritance pattern of the corresponding disease-causing genes (data not shown).

It was indicated that the novel de novo missense variation, TGFB2: p.L427P, was highly conserved among multiple species (Fig. 3C). Furthermore, in silico analysis results obtained using the SIFT (with the index of ‘0’) and PolyPhenV2 (with the index of ‘0.996’) softwares identified the variation as ‘deleterious/probably damaging’. Additionally, biophysical
analysis suggested that the helix in the kinase domain was damaged by the missense variant, the hydrogen bond force was reduced and the hydrophobic pocket was affected to some extent (Fig. 3d and e).

According to the 2019 American College of Medical Genetics and Genomics (ACMG) & Clinical Genome Resource guideline for CNV interpretation (21), the CNV detected, seq[GRCh37]del(12)(q24.21-q24.23)g.116399065-120555197, was interpreted as pathogenic with a score >1 (variant classification, 2A). Similarly, in accordance with the 2015 ACMG guideline for sequence variant interpretation (22), the TGFB R2:NM_001024847.2:exon5:c.1280T>c variant was deemed to be likely pathogenic (variant classification evidence, PS2+PM2+PP2+PP3).

Additionally, two pathogenic variations not associated with CTEV were detected, which were a paternal MYBPC3:NM_000256.3:exon6:c.769C>T: p.H257Y variation in case 4 and a maternal BRCA1:NM_007300.3:exon14:c.4497G>T: p.E1499D variation in case 5 (data not shown).

Discussion

CTEV seriously affects the aesthetics and functions of patients. The incidence and pathogenesis of the condition varies among different ethnicities, suggesting that genetic factors play an important role (23). I CTEV only follows the Mendelian inheritance pattern in minorities, including Polynesians (24), and therefore further investigation into the multifactorial pathogenesis of I CTEV in other ethnicities is required. Previous studies identified certain potential pathogenic genes primarily by single-nucleotide polymorphism typing combined with statistical analysis (2,11,25). Subsequently, further studies performed chromosomal microarray analysis and other methods to detect clinically significant CNVs containing important genes (7,26). However, few attempts have been made to apply next generation sequencing methods, including WES, to the identification of CTEV genetic variations (16,27).

A study conducted by Yang et al (28) identified variants in the filamin B gene, which is consistent with the findings of the present study (17). In the present study, the detection rate of variants was low for the majority of I CTEV cases, indicating that the pathogenesis of I CTEV in the Chinese population is relatively complicated and further suggesting multifactorial inheritance. Therefore, to explore the pathogenesis of I CTEV by detecting rare variants, an effective method would be to increase the sample size and subsequently perform gene ontology enrichment analysis (29).

The MED13L gene on chromosome 12q24 encodes a subunit of the large mediator complex that functions with DNA-binding transcription factors and RNA polymerase II for gene activation or repression (30). The gene is part of the evolutionarily conserved Thyroid Hormone Receptor Associated Protein gene family, which encode proteins that regulate embryonic development (31). Several studies have indicated that heterozygous loss of function variations resulting in haploinsufficiency could cause Mental Retardation and Distinctive Facial Features with or without cardiac defects [Mendelian Inheritance in Man (MIM), #616789] (32-35), and clubfoot was a notable phenotype in these cases. In the present study, it was hypothesized that the microdeletion of 12q in case 1 would result in MRFacd, particularly on the basis of the aforementioned evidence. Therefore, the mental and cardiovascular development of this infant should be closely monitored.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Sampling time (after birth)</th>
<th>Main manifestations</th>
<th>Family history</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Male</td>
<td>2 months</td>
<td>Bilateral CTEV; VSD and patent ductus arteriosus at 4 month follow-up</td>
<td>G1P1, no family history of CTEV</td>
</tr>
<tr>
<td>Case 2</td>
<td>Female</td>
<td>1 month</td>
<td>Bilateral CTEV</td>
<td>G2P1, onemiscarriage at early pregnancy (without genetic testing), no family history of CTEV</td>
</tr>
<tr>
<td>Case 3</td>
<td>Female</td>
<td>1 month</td>
<td>Bilateral CTEV</td>
<td>G1P1, no family history of CTEV</td>
</tr>
<tr>
<td>Case 4</td>
<td>Male</td>
<td>5 days</td>
<td>Unilateral (left) CTEV</td>
<td>G1P1, no family history of CTEV</td>
</tr>
<tr>
<td>Case 5</td>
<td>Female</td>
<td>15 days</td>
<td>Unilateral (left) CTEV</td>
<td>G2P2, one 3-year-old healthy son, no family history of CTEV</td>
</tr>
<tr>
<td>Case 6</td>
<td>Male</td>
<td>17 days</td>
<td>Unilateral (right) CTEV; arthrogryposis at 3 month follow-up</td>
<td>G1P1, no family history of CTEV</td>
</tr>
<tr>
<td>Case 7</td>
<td>Male</td>
<td>1 month</td>
<td>Bilateral CTEV</td>
<td>G2P2, one 4-year-old healthy daughter, no family history of CTEV</td>
</tr>
<tr>
<td>Case 8</td>
<td>Female</td>
<td>25 days</td>
<td>Unilateral (right) CTEV</td>
<td>G1P1, no family history of CTEV</td>
</tr>
<tr>
<td>Case 9</td>
<td>Male</td>
<td>1 month</td>
<td>Unilateral (right) CTEV</td>
<td>G1P1, no family history of CTEV</td>
</tr>
</tbody>
</table>

CTEV, congenital talipes equinovarus; VSD, ventricular septal defect; G, gravida; P, para.
The TGFBR2 (MIM, *190182) gene on chromosome 3p24 belongs to the serine-threonine kinase family (36). The activities of TGF-β1 (TGFB1; MIM, *190180) in regulating cell proliferation, differentiation and extracellular matrix production are mediated via these receptors. In the present study, the variant in case 3 was located in the protein kinase domain of TGFBR2. Previous studies have reported that missense variants occurring in this domain can cause Loeys-Dietz syndrome type II (37,38). Therefore, based on the results of genetic analysis, the child in case 3 should receive long-term follow-up in order to determine whether they display other phenotypes, as well as to provide further counseling and guidance for the subsequent pregnancies within the family. Moreover, although biophysical analysis predicted how the variant may alter the structure of TGFBR2, further studies are required to determine how the interaction between TGFBR2 and its receptor maybe affected.

According to the present study, the use of WES as a first-line method for the detection of ICTEV in children may not be efficient. However, WES provided ample sensitivity for the detection of rare syndromic CTEVs and consequently may be beneficial for the accurate differential diagnosis. The main limitations of the present study were the small sample size and the heterogeneity of CTEV. To validate the results of the present study, studies including larger and more well defined patient cohorts without congenital co-morbidities are required. Additionally, reporting the detection of pathogenic variants unrelated to proposed phenotypes presents an ethical challenge, which must be well communicated with patients and clearly reflected in informed consent.
In conclusion, the present study suggested that WES may serve as a comprehensive method for the detection of rare variants in coding sequences. In particular, WES displayed the advantage of detecting syndromic CTEV, which has a phenotype that is not easy to determine.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JZ and YL designed the current study. JZ and SL analyzed the data and drafted the manuscript. SM and YLiu recruited the case studies and performed the experiments. XW performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shijiazhuang Obstetrics and Gynecology Hospital (approval no. 20180015) and written informed consent was obtained from the parents of all patients.

Patient consent for publication

This study has followed the principles of anonymity; no direct or indirect identifiers of our participants were used for publication. Written informed consent was obtained for publication.
Competing interests
The authors declare that they have no competing interests.

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