Brief communication

Heterozygous deletion of *HOXC10-HOXC9* causes lower limb abnormalities in congenital vertical talus

Genes involved in limb patterning are sensitive to the gene dosage effect.[1] In this brief communication, we examine the possible associations between the heterozygous deletion of *HOXC10-HOXC9* and congenital vertical talus (CVT). In 2016, Alvarado *et al*[2] reported the associations between deletions of *5′ HOXC* genes (*HOXC12, HOXC13*) and CVT; however, subsequent reports on CVT-related genes have been limited. In this study, a novel 18.7 kb heterozygous deletion, which affected the *HOXC10* and *HOXC9* genes, was exclusively identified in CVT-affected patients. Additionally, in a mouse C2C12 cell model, we found that this deletion impaired cell proliferation and differentiation. Our findings contribute to existing knowledge on CVT-related genes, offering possibilities for genetic diagnosis of CVT.

CVT is a type of foot deformity, with an estimated prevalence of approximately 1 in 10 000 live births.[3] It is characterised by irreducible dislocation of the talus scaphoid joint, which leads to severe impairment of foot function and walking gait in children. In this study, we assessed 56 members from a family, which included 13 individuals affected with CVT and 43 unaffected individuals (figure 1A). All affected individuals in this family had lower limb deformities, notably protruding plantars and extreme stiffness in the ankle joints, which caused steppage gait and impaired mobility during activities such as running and climbing stairs (figure 1B). There was no upper limb deformity or intellectual disability among the individuals. Deformities were classified as severe, moderate or mild depending on the severity. Muscle abnormalities in the affected individuals included muscle atrophy in the lower limbs and flexor weakness in the feet. Babinski's and Oppenheim's signs were negative. The proband (III-11) exhibited the most severe phenotypes, presenting multiple deformities including CVT, Charcot-Marie-Tooth (CMT), scoliosis (online supplemental figure 1) and hip dislocation. Other affected individuals also exhibited sporadic phenotypes (table 1).

Radiography and CT examinations revealed talus–navicular joint dislocation accompanied by rigidity and claw-shaped changes in the phalanges among the affected individuals (figure 1C). Through measurement on lateral radiographs with the foot positioned neutrally or in plantar flexion, we observed a significant increase in rigidity at the talar and calcaneal axis–first metatarsal base angles, which aligned with the established criteria for diagnosing vertical talus.[4] Limb CT of patient III-11 revealed decreased lower limb muscle volume on both sides, demonstrating significant atrophy in the gastrocnemius and fibula muscles. The extent of atrophy was substantial enough to result in the replacement of the peroneus brevis and peroneus longus by fat in the transverse view (online supplemental figure 2). Ultrasonography of individual IV-12 revealed the appearance of a rocker-bottom foot at 23 weeks of gestation (figure 1D). Electromyograms (EMGs) of patients III-11 and IV-9 revealed peripheral nerve damage in both lower limbs. Denervation potentials were observed, and motor unit potentials were reduced. The motor nerve amplitudes of the common peroneal and tibial nerves were significantly reduced (>80%), whereas the conduction velocity was normal (>38 m/s), as expected from CMT II. No abnormalities were observed in the peripheral nerves of the upper limbs. H&E staining of the gastrocnemius muscle revealed no abnormalities (online supplemental figure 3).

The linkage analysis of 11 individuals revealed a region on chromosome 12 with logarithm of the odds (LOD) scores >2 (online supplemental figure 4). We performed a genome-wide analysis of CNVs in the affected individuals (patients II-15 and IV-9) using Agilent 1×1 M array comparative genomic hybridisation after obtaining negative whole-exome sequencing results. We identified a heterozygous deletion within the human chromosome 12q13.13 (figure 1E), located in the region where the LOD scores exceeded 2. This deletion ((GRCh38) chr12:53982209–54000956) was affected by both *HOXC9* and *HOXC10*, and it cosegregated with CVT in the family (figure 1F). To date, no genic or exonic deletions in the *HOXC* cluster have been reported in the human population genome database gnomAD (V.3.1.1) or in the pathogenic variant databases ClinVar and DECIPHER. At the deletion junction, microhomology of 3 bp (CAG) was observed (online supplemental figure 5), providing evidence to support the mechanism of non-homologous DNA end joining.[5]

Furthermore, we used mouse C2C12 myoblast cells as a model to explore the pathogenic function in the musculoskeletal system. *HOXC9* and *HOXC10* are conserved between mice and humans. Using epiCRISPR/Cas9 technology, the cell clone C1 with a heterozygous deletion ((GRCm39)chr15:102873360–102890367) affecting both *Hoxc9* and *Hoxc10*, that is, *+/Del(Hoxc10-Hoxc9)*, was selected for subsequent assays (figure 1G).

The relative mRNA and protein expression levels of both *Hoxc9* and *Hoxc10* were decreased by approximately half in *+/Del* C2C12 cells (figure 1H). Both EdU and CCK8 proliferation assays showed a significantly reduced proliferation capacity in *+/Del* C2C12 cells (figure 1I and online supplemental figure 6). The immunofluorescence analysis of *Myhc*[6] in *+/Del* C2C12 cells revealed a significantly lower fusion index each day following induction of differentiation, indicating a diminished capacity for muscle bundle differentiation (online supplemental figure 7).

RNA-Seq was performed on *+/Del* C2C12 cells and wildtype (WT) cells. 2489 differentially expressed genes were identified, of which 1288 were upregulated genes and 1201 were downregulated genes. The top 20 enriched pathways are shown in figure 1J. As expected, the pathways associated with muscle fibre transformation, such as 'calcium signaling pathway',[7] 'mitogen-activated protein kinases (MAPK) signaling pathway'[8] and 'extracellular matrix-receptor (ECM-receptor) interaction pathway',[9] were significantly enriched. The expression of some essential genes was consistent with the real-time PCR results. The neurofilament light chain (*Nefl*), which is a serum biomarker of neuronal injury, was significantly downregulated.[10] *Myh4* and *Ccnd2* (the key regulators of terminal differentiation of muscle progenitor cells) were also downregulated. The *Smad* family members, which transduce signals from the transforming growth factor-beta (TGF-beta) family, were significantly upregulated. The intersections of genes that play key roles in the differentiation of myoblasts into bundles were also affected, particularly nerve growth factor (*Ngf*) (online supplemental figure 8), which was enriched in the four pathways associated with skeletal muscle development. Transcription factors (TFs) are located upstream of the transcriptional regulatory networks. As shown in online supplemental figure 9, the *Hox* family was the second most significantly enriched TF family. Taken together, these findings further indicate that the heterozygous deletion of *Hoxc10-Hoxc9* impairs the differentiation and maturation capacity of C2C12 myoblasts by disturbing multiple pathways associated with skeletal muscle development.

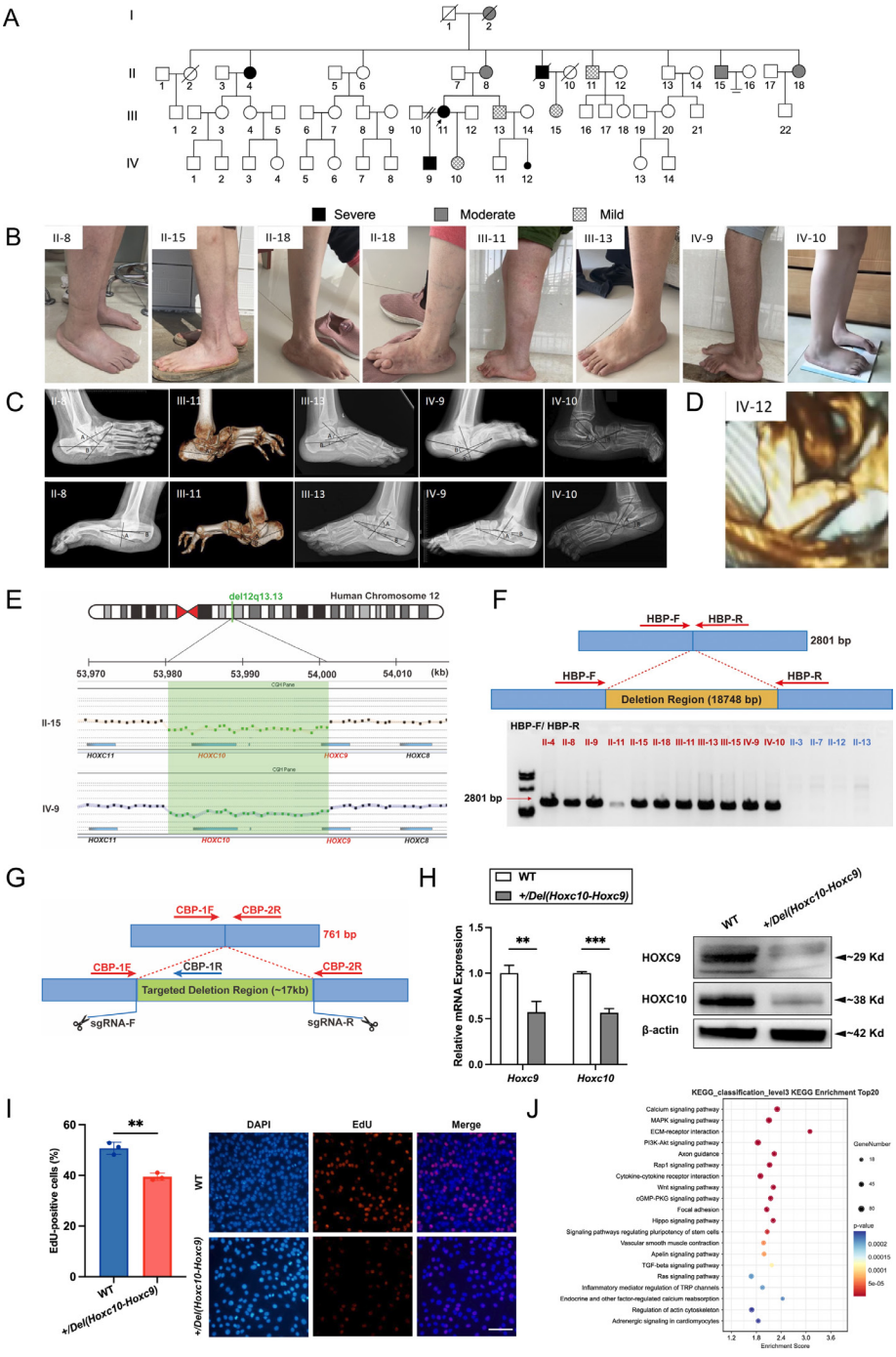

Figure 1 (A) Pedigree of the family with congenital vertical talus, including 13 affected individuals with varying degrees of severity. (B) Muscle atrophy features observed in the lower limbs of the affected individuals. (C) Lateral radiographs with the feet in the neutral position or plantar flexion revealed that the TAMBA and the CAMBA were significantly increased (A: TAMBA, B: CAMBA). (D) Ultrasound of the fetus (patient IV-12) showed the appearance of a rocker-bottom foot at 23 weeks of gestation. (E) An 18.7 kb heterozygous deletion was identified on 12q13.13 in the affected individuals (patients II-15 and IV-9) using CGH microarrays. The deletion region is denoted by the green shadow. (F) Schematic illustration of the *HOXC9-HOXC10* deletion and the products of amplification. The amplification product generated by a primer pair (HBP-F/R) of size 2.8 kb (indicated by the red arrow) was present in all affected individuals (labelled in red) but absent in all unaffected individuals (labelled in blue, representing a subset of individuals). (G) Schematic illustration of the deletion by epiCRISPR/Cas9 and the amplification products in mouse C2C12 cell model. The amplification product generated by the CBP-1F/2R primer pair was 761 bp in size (indicated by the red arrow), and the product generated by the CBP-1F/1R primer pair was 650 bp (indicated by the blue arrow) in *+/Del(Hoxc10-Hoxc9)* C2C12 cells. (H) The relative mRNAs and protein levels of *Hoxc9* and *Hoxc10* were reduced by half in the *+/Del* C2C12 cells (**p<0.01, ***p<0.001). (I) The proliferation capacity of *+/Del* C2C12 cells, assessed via EdU staining, was significantly lower than that of WT cells. EdU staining is depicted in red, and nuclei are stained blue (Hoechst 33342) (scale bar=200 µm). The percentage of EdU-positive cells was significantly reduced as quantified using the ImageJ software (**p<0.01). (J) The top 20 pathways of DEGs. The x-axis represents the number of DEGs enriched in each. The primer Sequences (HBP-F/R, CBP-1F/1R/2R) showed in online supplemental table 1. CAMBA, the calcaneal axis–first metatarsal base angle; CGH, comparative genomic hybridisation; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; TAMBA, the talar axis–first metatarsal base angle; WT, wildtype.

Table 1 Clinical features of affected individuals in the four-generation family

Patients	Age range (years)	Rocker-bottom foot	Folded toes	Fourth metatarsal short	CMT	Stiff ankle flexibility	Hip dislocation	Scoliosis
I-2*	–	+	+		+	+		+
II-4	66–70	+	+		+	+		+
II-8	61–65	+	+		+	+		
II-9*	–	+			+	+		
II-11	56–60	+			+	+		
II-15	51–55	+	+		+	+		
II-18	46–50	+	+	+	+	+		
III-11	36–40	+			+	+	+	+
III-13	36–40	Flat foot	+		+	+		
III-15	26–30	+			+	+		
IV-9	16–20	+ (left), flat foot (right)			+	+		+
IV-10	11–15	+ (left), clubfoot (right)	+		+	+		+
IV-12*	–	+						

*The patients have passed away and their phenotypes were described by relatives.
CMT, Charcot-Marie-Tooth disease.

In conclusion, based on genetic and functional data, our findings support the hypothesis that the heterozygous deletion of *HOXC10-HOXC9* can induce CVT and lower limb deformities. Furthermore, our findings offer crucial insights for genetic counsellors and clinicians, enhancing their understanding of the genetic causes of CVT and enabling the development of personalised treatment strategy.

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Acknowledgements We thank the family members for their participation in this investigation. And we thank Prof. Feng Zhang and Prof. Yongming Wang at Fudan University for their technological supports.

Contributors LC, SZ and XL contributed to the overall planning and reporting of the work described in the article. LC, ZY, JG and XL contributed to sample collection and processing and conducted the clinical evaluations. LC and SZ performed the genetic analysis and wrote the manuscript. SZ performed the cell model and functional experiments. WS and LW participated in the ethical applications and manuscript revisions. LC and XL are guarantors and accept full responsibility for the finished work and/or the conduct of the study, had access to the data and controlled the decision to publish. All authors contributed to the review and final approval of the manuscript.

Funding This investigation was supported by the Scientific Research Project from the Health Commission of Shanxi Province (2021014).

Competing interests None declared.

Patient consent for publication Consent obtained directly from all individuals or legal guardians of minors.

Ethics approval This study involves human participants and was approved by the Institutional Ethics Committee at Changzhi Maternal and Child Health Care Hospital (CZSFYLL2021-005). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136jmg-2023-109656).

LC and SZ contributed equally.

To cite Chen L, Zhao S, Song W, *et al*. *J Med Genet* 2024;**61**:777–779.

Received 22 September 2023
Accepted 2 January 2024
Published Online First 31 January 2024

J Med Genet 2024;**61**:777–779.
doi:10.1136/jmg-2023-109656

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