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ORIGINAL INVESTIGATION

Case series of congenital pseudarthrosis of the tibia unfulfilling neurofibromatosis type 1 diagnosis: 21% with somatic *NF1* haploinsufficiency in the periosteum

Yu Zheng[1,2,3] · Guanghui Zhu[2] · Yaoxi Liu[2] · Weihua Zhao[2] · Yongjia Yang[2] · Zhenqing Luo[2] · Yuyan Fu[2] · Haibo Mei[2] · Zhengmao Hu[1,3]

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Abstract

Up to 84% of patients with congenital pseudarthrosis of the tibia (CPT) present with neurofibromatosis type 1 (NF1) (NF1-CPT). However, the etiology of CPT not fulfilling the NIH diagnostic criteria for NF1 (non-NF1-CPT) is not well understood. Here, we collected the periosteum tissue from the pseudarthrosis (PA) site of 43 non-NF1-CPT patients and six patients with NF1-CPT, together with the blood or oral specimen of trios (probands and unaffected parents). Whole-exome plus copy number variation sequencing, multiplex ligation-dependent probe amplification (MLPA), ultra-high amplicon sequencing, and Sanger sequencing were employed to identify pathogenic variants. The result showed that nine tissues of 43 non-NF1-CPT patients (21%) had somatic mono-allelic *NF1* inactivation, and five of six NF1-CPT patients (83.3%) had bi-allelic *NF1* inactivation in tissues. However, previous literature involving genetic testing did not reveal somatic mosaicism in non-NF1-CPT patients so far. In NF1-CPT patients, when the results from earlier reports and the present study were combined, 66.7% of them showed somatic *NF1* inactivation in PA tissues other than germline inactivation. Furthermore, no diagnostic variants from other known genes (*GNAS*, *AKT1*, *PDGFRB,* and *NOTCH3*) related to skeletal dysplasia were identified in the nine *NF1* positive *non-NF1-CPT* patients and six NF1-CPT patients. In conclusion, we detected evident somatic mono-allelic *NF1* inactivation in the non-NF1-CPT. Thus, for pediatric patients without NF1 diagnosis, somatic mutations in *NF1* are important.

Abbreviations

CPT	Congenital pseudarthrosis of the tibia
NF1	Neurofibromatosis type 1
NF1-CPT	CPT fulfilling the NIH diagnostic criteria for NF1
Non-NF1-CPT	CPT not fulfilling the NIH diagnostic criteria for NF1
MNF1	Mosaic NF1
MLPA	Multiplex ligation-dependent probe amplification
PA	Pseudarthrosis
WES	Whole-exome sequencing
CNV	Copy number variation
CAL	Café au lait macules
PBS	Phosphate-buffered saline
SNV	Single-nucleotide variations

✉ Haibo Mei
meihaiboprof@outlook.com

✉ Zhengmao Hu
huzhengmao@sklmg.edu.cn

1 Center for Medical Genetics and Hunan Key Laboratory of Medical Genetics, School of Life Sciences, Central South University, Changsha, Hunan, China

2 Department of Pediatric Orthopedics and Pediatrics Research Institute of Hunan Province, Hunan Children's Hospital, the Pediatric Academy of the University of South China, 86 Ziyuan Road, Changsha, Hunan, China

3 Hunan Key Laboratory of Molecular Precisional Medicine, Central South University, Changsha, Hunan, China

Introduction

Congenital pseudarthrosis of the tibia (CPT) is a rare disease characterized by recalcitrant healing of the tibia followed by anterolateral bowing, pathological fractures, or pseudarthrosis in early life (Hefti et al. 2000; O'Donnell et al. 2017; Vander Have et al. 2008). Up to 84% of patients with CPT match the NIH diagnostic criteria for neurofibromatosis type 1 (NF1, MIM# 162200) (Neurofibromatosis 1988; Van Royen et al. 2016). NF1 is an autosomal-dominant genetic

disease involving cutaneous, ocular, or osseous lesions, and some of the affected individuals have neurological, vascular, or cardiac manifestations. NF1 occurs in approximately 1/3000 to 1/2000 births (Evans et al. 2010; Lammert et al. 2005; Uusitalo et al. 2015), and < 5% of patients with NF1 have CPT (Friedman and Birch 1997; Schindeler and Little 2008; Young et al. 2002).

Haplo-insufficiency of the *NF1* gene brought by loss-of-function (LoF) variation can cause NF1. *NF1* is a tumor suppressor that encodes neuro-fibromin activating ras GTPase to control cellular proliferation, and loss of neuro-fibromin activates the RAS/MAPK signaling pathway leading to cell overgrowth (Andersen et al. 1993; Ballester et al. 1990; Basu et al. 1992; Bollag et al. 1996; Cichowski and Jacks 2001; DeClue et al. 1991; Lau et al. 2000; Xu et al. 1990a, b). Previous studies that detected the CPT pseudarthrosis (PA) tissue focused on CPT patients with NF1 (NF1-CPT) (Brekelmans et al. 2019; Lee et al. 2012; Margraf et al. 2017; Paria et al. 2014; Sakamoto et al. 2007; Sant et al. 2015; Stevenson et al. 2006; Tahaei et al. 2018), and molecular testing mainly targeted the DNA, RNA, or protein of *NF1* via techniques, such as whole-exome sequencing (WES), RNA-seq, protein truncation test, multiplex ligation-dependent probe amplification (MLPA), gene-targeted microarray, quantitative PCR, and long-range PCR. However, approximately 26% of CPT cases do not meet NF1 diagnosis (non-NF1-CPT), and no genetic findings have been reported. Thus, the non-NF1-CPT etiology is still not understood.

Non-NF1-CPT patients usually have an isolated phenotype in the tibia. Generally, non-NF1-CPT has similar clinical manifestations as NF1-CPT (Zhu et al. 2019), including the time of tibia bowing or fracture onset, lateralization, abnormality of the proximal tibial epiphysis, and tibial union follow-up after surgery. Previous histological, pathological, and biological studies found no significant differences between NF1-CPT and non-NF1-CPT (Granchi et al. 2010; Hermanns-Sachweh et al. 2005), with both showing thickened periosteum with nerve cells accumulating around small arteries (Hermanns-Sachweh et al. 2005). In addition, a biological study using cultured bone marrow stromal cells from the PA site showed reduced osteogenicity in NF1-CPT and non-NF1-CPT than in controls (Granchi et al. 2010). However, our previous study of 75 patients with CPT identified no germline pathogenic *NF1* variants in all 20 non-NF1-CPT patients (Zhu et al. 2019). In the reviewed 158 cases with mosaic NF1 (MNF1), two cases presented non-NF1-CPT but did not undergo genetic testing (El-Rosasy 2020; Garcia-Romero et al. 2016; Listernick et al. 2003).

Periosteum covering the outer surface of bone is essential for skeletal development and growth, and it also works as a central mediator of bone-healing after fracture (Hutmacher and Sittinger, 2003; Ozaki et al. 2000; Roberts et al. 2015). Pathologically, both NF1-CPT and non-NF1-CPT showed thicken periosteum with fibrous hyperplasia at the PA site (Ippolito et al. 2000; Sakamoto et al. 2007; Zhu et al. 2019). For NF1-CPT, some studies found bi-allelic *NF1* inactivation (also known as second hit, double inactivation) in the PA tissue, which covered approximately 65% of the published cases (Lee et al. 2012; Paria et al. 2014; Sakamoto et al. 2007; Sant et al. 2015; Stevenson et al. 2006). However, for non-NF1-CPT, as no germline *NF1* inactivation has been reported, we supposed that somatic *NF1* inactivation could exist in the pathological periosteum at PA site related to CPT lesion. Here we obtained the DNA of the PA periosteum of 43 non-NF1-CPT patients and six patients with NF1-CPT and performed whole-exome plus copy number variation (CNV) sequencing (WES + CNV) and MLPA to analyze pathogenic variants spanning the known associated genes. We then validated the identified pathogenic variants using DNA from the trios (affected probands and unaffected parents).

Methods

Aim, design, and settings

This study aimed to investigate disease-causing variants in non-NF1-CPT patients with unknown etiologies. Toward this goal, using the PA tissues, we performed WES + CNV and MLPA in 43 non-NF1-CPT patients to first screen for known disease-causing genes. The source of mutation was then identified using trios for precise diagnosis, management, and genetic counseling. Concurrently, the six NF1-CPT patients were analyzed using the same pipeline for comparison.

Participants

After obtaining ethical approval and informed consent, we studied 43 patients with non-NF1-CPT and six patients with NF1-CPT who underwent surgery (Supp. Table S1). The patients were not diagnosed with osteofibrous dysplasia. We collected detailed clinical information, family history, and periosteum of the discarded PA tissue of each patient (Table 1). The peripheral blood or oral samples of eighteen trios (affected and unaffected parents) were also collected. According to the NF1 diagnostic criteria (NIH, 1988) (Neurofibromatosis 1988), all non-NF1-CPT cases have an isolated phenotype of tibial pseudarthrosis (HP:0009736) and do not match NF1. All six NF1-CPT patients had six or more café au lait macules (CAL, HP:0007565) > 5 mm in greatest diameter and were diagnosed with NF1 (Table 1).

Table 1 CPT patient information and genetic findings of *NF1*

Case ID		Phenotype				Inheritance				Genotype				
	Sex[a]	Time of tibia bowing or facture onset[b]	CPT lateralization	Match NF1?	Other phenotype	Family history	PA periosteum zygosity (ref/alt, VAF)	PBL/ oral cells zygosity	Inheritance mode	Nucleotide change (NM_000267.3)	Amino acid change (NP_000258.1)	ACMG criteria[c]	Novel/ known variation	Validated Source
P1	F	4 m	Right	Non-NF1	No	No	Het	Normal	Somatic	c.383_8903del	Entire loss	P	ClinVar	Tissue
P2	M	Birth	Left	Non-NF1	No	No	Het (55/125, 69%)	Normal	NA	c.1185+1G>A	p.?	P	ClinVar	Tissue
P3	M	Birth	Left	Non-NF1	No	No	Het (100/136, 58%)	Normal	Somatic	c.4269+2 T>C	p.?	P	ClinVar	Tissue, twins+parents oral samples
P4	F	5 m	Right	Non-NF1	No	No	Het (16/17, 52%)	Normal	Somatic	c.484C>T	p.(Gln162Ter)	P	HGMD	Tissue, trio oral samples
P5	M	NA	Left	Non-NF1	No	No	Het (72/138, 66%)	Normal	NA	c.3897del	p.(Lys1299Asnfs-Ter10)	P	ClinVar	Tissue
P6	M	Birth	Right	Non-NF1	No	No	Het (50/103, 67%)	Normal	Somatic	c.205-1G>T	p.?	P	Novel	Tissue, trio oral samples
P7	M	8 m	Right	Non-NF1	No	No	Het (138/140, 50%)	Normal	Somatic	c.2002-1G>C	p.?	P	Novel	Tissue, patient oral samples
P8	M	11 m	Left	Non-NF1	No	No	Het (28/70, 71%)	Normal	Somatic	c.4173_4174del	p.(Arg1391Serfs-Ter11)	P	Novel	Tissue, trio oral samples
P9	F	Birth	Left	Non-NF1	No	No	mosacism (WES: 95/23, 19%; UHAS: 62,634/12288, 16%)	Normal	Somatic	c.334C>T	p.(Gln112Ter)	P	HGMD	Tissue, trio PBL
P10	F	Birth	Right	NF1	CAL	Twin sister	Het (54/43, 44%)	Het	Germline denovo	c.4267A>G	p.(Lys1423Glu)	P	ClinVar	Tissue, twins+parents PBL
P11	M	Birth	Left	NF1	CAL	No	Hom (WES: 719/234, 25%; Sanger: hom)[d]	Het	Germline denovo	c.4120_4123del	p.(Gln1374Valfs-Ter10)	P	Novel	Tissue, twins+parents PBL
P12	M	Birth	Right	NF1	CAL	No	Hom (WES: 17/162, 91%, Sanger: hom)	Het	Germline denovo	c.6709C>T	p.(Arg2237Ter)	P	ClinVar	Tissue, trio oral samples
P13	M	3 m	Right	NF1	CAL	Mother	Hom (WES: 2/4, 67%; Sanger: hom)	Het	Germline maternal	c.31C>T	p.(Gln11Ter)	P	ClinVar	Tissue, patient+father oral samples
P14	M	19 m	Left	NF1	CAL	Mother	Het (134/126, 48%)	Het	Germline maternal	c.4981_4982del	p.(Cys1661Ter)	P	Novel	Tissue, trio oral samples

Table 1 (continued)

Case ID	Phenotype				Inheritance				Genotype				
	Sex[a]	Time of tibia bowing or facture onset[b]	CPT lateralization	Match NF1?	Other phenotype	Family history	PA periosteum zygosity (ref/alt, VAF)	PBL/ oral cells zygosity	Inheritance mode	Nucleotide change (NM_000267.3)	Amino acid change (NP_000258.1)	ACMG criteria[c]	Novel/ known variation	Validated Source
							Mosacism (123/62,34%)	Normal	Somatic	c.989C > A	p.(Ala330Glu)	LP	ClinVar VUS	Tissue, trio oral samples
P15	F	22 m	Right	NF1	CAL	No	Het (142/134, 49%)	Het	Germline denovo	c.6162G > A	p.(Trp2054Ter)	P	Novel	Tissue, trio oral samples
							Mosacism (282/63, 18%)	Normal	Somatic	c.1381C > T	p.(Arg461Ter)	P	ClinVar	Tissue, trio oral samples

CAL multiple Cafe-au-lait macules (HP:0007565), *NA* not available, *VUS* Variant with uncertain significance, *Ref/alt* depth of reference allele/depth of alternative allele, *VAF* variant allele frequency, *UHAS* ultra-high amplicon sequencing

[a] *F* female, *M* male for sex

[b] *y* year, *m* month for age

[c] *P* pathogenic, *LP* likely pathogenic

[d] This variant was identified as low VAF in WES, and validated as homozygous in the patient's periosteum by Sanger sequencing (Supplementary Fig. S2)

Sample preparation and DNA extraction

The pathological periosteum surrounding the cortical bone of the discarded PA tissue during surgery (Supp. Fig. S1) was cut into pieces. No bone spicules were embedded. Each sample was approximately 200 mg and had a diameter of 5 mm approximately. One piece of periosteum biopsies was performed pathology detection and stained with hematoxylin and eosin (H&E) following the standard protocol. Other pieces of periosteum tissue were washed with phosphate-buffered saline (PBS), and then were frozen at − 80 °C or preserved in liquid nitrogen. Genomic DNA was extracted using the following steps: (1) the preserved periosteum was ground using the automatic sample quick grinding machine JXFSTPRP-12/16 (Tissuelyser, Shanghai, China; the ground parameter was 65 Hz for 60 s); (2) 500 µl Sodium Chloride–Tris–EDTA (STE) buffer solution (0.1 mol/L NaCl, 10 mmol/L Tris–Cl with pH = 8.0, 1 mmol/L EDTA), 10 mg/ml Proteinase K and 75 µl 10% SDS were added and digested over 24 h, (3) centrifugation and the supernatant were kept to extract DNA using the standard phenol–chloroform method, (4) the extracted DNA was then evaluated by agarose gel electrophoresis and quantified using Qubit 3.0. The genomic DNA of peripheral blood lymphocytes was extracted using the standard phenol–chloroform method. In addition, the genomic DNA of saliva or oral swabs was extracted using the Saliva DNA Purification Kit (Simgen, Hangzhou, Zhejiang, China) and Magbead Swab DNA Kit (CWBIO, Taizhou, Jiangsu, China), respectively.

Whole exome plus CNV sequencing and bioinformatics analysis

Forty-nine periosteal DNA samples and twelve blood DNA samples of unaffected parents were submitted to whole exome plus CNV sequencing (Supp. Table S1). Genomic DNA (200 ng) of each sample was fragmented, and the exome was captured using the AIExomeV1-CNV Enrichment Kit (iGeneTech, Beijing, China, capture region size: 62 Mb) for library preparation (concentration > 25 ng/ul, central peak 220–320 bp). The captured DNA was sequenced with 2 × 150 bp reads using an Illumina NovaSeq 6000 system (Illumina, San Diego, California, USA) following the manufacturer's instructions. Each sample yielded over 15 GB of raw data with a minimum mean depth of coverage of 100×. The average base Phred quality was over 36.1 (34.9–36.4), and the mean target coverage at 20X was 97.6% (84.4–99.4%).

The sequenced raw reads in FastQ file format were preprocessed using trim_galore (version 0.6.4, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove adapter-contaminated ends and low-quality bases with Phred scores < 20. Reads with > 5N bases, > 40% low-quality

bases, or trimmed lengths < 30 bp were also removed. Subsequently, the clean reads were mapped to the human reference sequence (version: GRCh37) using the alignment tool Burrows–Wheeler Aligner (BWA, Version 0.7.17-r1188) (Li and Durbin 2010). SAMtools (Li et al. 2009) and SAMBLASTER (version 0.1.26) (Faust and Hall 2014) were used to remove duplicate reads. The Genome Analysis Toolkit (GATK, version 3.8) (DePristo et al. 2011) refined the alignment and call variants.

SNV and InDel analysis

The generated single-nucleotide variations (SNVs) and short insertions and deletions (InDels) from GATK with genotype quality ≥ 20, depth ≥ 5, and variant allele frequency ≥ 0.15 were kept for subsequent analysis. They were annotated using ANNOVAR (Wang et al. 2010) and InterVar (version 20,180,118) (Li and Wang 2017). We removed the SNPs and InDels with population frequency (minor allele frequency, MAF) > 0.001 in gnomAD (East Asian and popmax of continental populations using WES and whole genome sequencing) and ExAC (all populations and East Asian) databases (Karczewski et al. 2017, 2020). SNPs and InDels shared by at least two of the 12 CPT family controls or shared by less than two of our in-house 528 WES family controls (with MAF > 0.001) (Liu et al. 2021; Yang et al. 2019) were also removed. The remaining non-benign heterozygous variants annotated by InterVar or ClinVar (version 20180603) in the coding or UTR regions were then used for further analysis. The prioritized variants of the *NF1* gene were screened using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD databases (public version, http://www.hgmd.cf.ac.uk) for known pathogenic records. VarSome (Kopanos et al. 2019) was employed to classify the pathogenicity of each variant according to the American College of Medical Genetics and Genomics (ACMG) criteria (Richards et al. 2015). Protein domains and repeats and homologous super-families of neuro-fibromin were queried from InterPro (http://www.ebi.ac.uk/interpro). In addition, the known disease-causing genes *GNAS*, *AKT1*, *PDGFRB,* and *NOTCH3*, related to musculoskeletal dysplasia similar to CPT, were screened for differential diagnosis.

Detection of CNVs and structural variations (SVs)

CNVkit (version 0.9.6) (Talevich et al. 2016) was used to detect CNVs in each patient. Twelve healthy parents were used as reference, and the threshold parameter "$-t = -1.1, -0.4, 0.3, 0.7$" was used for CNV calling. We then annotated the identified CNVs using ANNOVAR against the refGene, cytoBand, wgRna, genomicSuperDups, genomicCatalog, and CNVD databases. The detected CNVs overlapping *NF1*, *GNAS*, *AKT1*, *PDGFRB*, and *NOTCH3* genes were interpreted according to the ACMG and ClinGen technical standards (Riggs et al. 2020). Pathogenic or likely pathogenic CNVs were further validated using MLPA.

SVs were detected using the LUMPY-sv (version v0.3.0) (Layer et al. 2014). After filtering out the SVs shared by the 12 unaffected parents, the remaining SVs overlapping *NF1*, *GNAS*, *AKT1*, *PDGFRB*, and *NOTCH3* genes were subjected to for further validation. Integrative Genomics Viewer (IGV) (Thorvaldsdottir et al. 2013) was employed to view the variant-located chromosomal region using the patient's bam file to confirm the CNVs and SVs.

Validation by Sanger sequencing

The identified pathogenic or likely pathogenic, and LoF (nonsense, frameshifting indels and canonical splice-site) SNV and InDels that overlapped with the *NF1* gene were validated using Sanger sequencing in the trios [PA tissue of probands, peripheral blood, or saliva or oral swabs of probands and their parents (see Table 1 and Supp. Fig. S2 and Supp. Fig. S3 for details)]. First, we employed the Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design PCR primers (primers for validating SNPs and InDels were provided in Supp. Table S2). Subsequently, all variants were validated via independent PCR amplification and bidirectional DNA sequencing suing ABI 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Multiplex ligation-dependent probe amplification (MLPA)

For suspected CNVs overlapping the *NF1* gene, MLPA was performed to validate deletions or duplications encompassing ≥ 1 *NF1* exons or the entire gene. We used the SALSA MLPA probe P081 NF1 mix 1 and P082 NF1 mix 2 (MRC-HOLLAND, Amsterdam, Netherlands) to detect CNVs in the extracted DNA and performed dosage analysis following the manufacturer's instructions.

Mosaic variant validation using ultra-high amplicon sequencing

NF1-related pathogenic or likely pathogenic SNVs and InDels with allele fractions ≥ 5% and ≤ 30% were considered to suspect mosaic variations. Only one variant c.334C > T with an allele fraction of 18%, was used for further validation. The Primer-BLAST program was used to design PCR primers specific for the variant. The PCR product with the target length (699 bp) using the extracted tissue DNA was submitted for ultra-high amplicon sequencing. The target-captured DNA was then sequenced with 2 × 150 bp reads using an Illumina NovaSeq 6000 system (Illumina,

San Diego, California, USA) following the manufacturer's instructions. Furthermore, 1.32 Gb of raw data was obtained with a minimum mean depth of coverage over 70,000 × and an average base quality of 36. The raw reads were polished and then aligned to the human reference sequence (version: GRCh37) using BWA MEM. Variants in the target regions were identified using GATK. IGV was deployed to confirm the variants using the generated bam file.

Literature search

The literature search in PubMed was performed using the search terms "pseudarthrosis AND (tibia OR tibial) AND (NF1 OR neurofibromatosis) AND (genetic OR molecular)". Sixty-four articles that were published by September 30, 2021 were reviewed. Further filtering was done using keywords "somatic OR mosaic OR segmental OR localized" for non-NF1-CPT reports. We further searched for publications on NF1-CPT with somatic bi-allelic *NF1* inactivation using the keywords "second hit", "biallelic", or "double inactivation". We then manually screened these publications with clinical features and *NF1* variants reported in patients.

Results

Non-NF1-CPT patients have pathogenic somatic *NF1* variants of in the PA periosteum

After quality and allele frequency filtration, 742 pathogenic, likely pathogenic or LoF SNPs and InDels were left in the WES data of all the 43 non-NF1-CPT and 6 NF1-CPT PA periosteal samples. They were distributed in 631 genes, and the *NF1* gene covered a significantly higher proportion (14/49; 28.6%) of the sequenced cases than other genes (< 10% each). In the PA periosteum tissue of 43 non-NF1-CPT patients, 9 (21%, 9/43) were identified *NF1* heterozygous variants (Fig. 1). All nine variants were classified as pathogenic based on the ACMG criteria. Further by validating the variants in family trios, seven of nine variants were found in the PA periosteum but not in the peripheral blood or oral epithelial cells of patients, or their parents (Table 1, Supp. Fig. S1). Therefore, the seven variations were confirmed to be somatic. For example, P1 was identified a heterozygous *NF1* gene loss by MLPA in the PA tissue. P3 has ClinVar known pathogenic splicing variant c.4269 + 2T > C. P4 has c.484C > T (p.Gln162Ter) leading to premature translation termination. P9 was detected a mosaic c.334C > T (p.Gln112Ter) in the PA periosteum. The three variants were recorded in the ClinVar or HGMD databases as pathogenic. In addition, P6 and P7 harbor novel splicing variants c.205-1G > T and c.2002-1G > C; while P8 has a novel frameshift deletion variant c.4173_4174del (p.Arg1391SerfsTer11).

In case of the other two variants, which are loss of contact and lack of family samples, it was not confirmed whether c.1185 + 1G > A in P2 and c.3897del (p.Lys1299AsnfsTer10) in P5 were germline or somatic.

Bi-allelic *NF1* inactivation occurs in the PA site of patients with NF1-CPT

Using the same pipeline, all six patients with NF1 identified pathogenic *NF1* variants, and three had bi-allelic *NF1* inactivation in the periosteum of the PA site (Table 1, Supp.

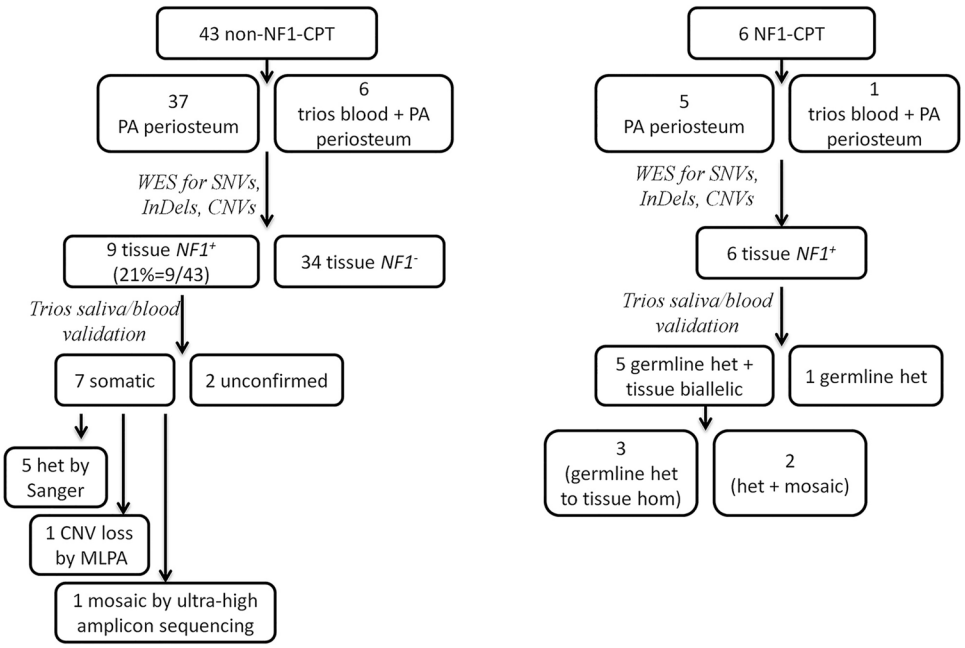

Fig. 1 Strategy and genetic analysis overview of two groups of CPT patients in this study. het: heterozygous. hom: homozygous

Fig. S2). Patients P11, P12, and P13 were found heterozygous *NF1* pathogenic variants in the blood or oral epithelial cells (p.Gln1374ValfsTer10 for P11, p.Arg2237Ter for P12, and p.Gln11Ter for P13), whereas the three variants were homozygous in the PA periosteum. All three variants were related to premature termination of translation. Among them, the variants in P11 and P12 were not found in their unaffected parents and were confirmed to be de novo. Another variant in P13 was confirmed to be maternally inherited and the patient's mother had NF1 findings. Notably, the InDel variant in P11's tissue was identified as heterozygous with low-variant allele frequency (719/234; 25%) by WES, while by Sanger sequencing, this variant was homozygous in the patient's PA periosteum and heterozygous in the patient's blood. This variant was not detected in P11's father and mother by Sanger sequencing. Moreover, the tibia of P11 and P13 were not un-ioned after surgery (Supp. Table S1). We supposed that one of the reasons was non-autogenous iliac bone graft applied.

Specifically, patient P10 was the only mono-allelic variant c.4267A > G (p.Lys1423Glu) identified in blood and PA periosteum. The variant was classified as pathogenic based on the ACMG criteria and is a known pathogenic variant in ClinVar. This variant was also identified in P10's monozygotic twin sister, who also had NF1 but without CPT manifestations. The patient's parents did not have the variant, suggesting that it is de novo nature.

Somatic mosaicism in PA periosteum happens in both non-NF1-CPT and NF1-CPT

In non-NF1-CPT, patient P9 was identified a mosaic c.334C > T (p.Gln112Ter) with an allelic fraction of ~ 16% in the PA tissue but not in the blood (Table 1). WES and ultra-high amplicon sequencing demonstrated similar allelic fractions of 19% (depth: 23/118) and 16% (depth: 12,288/74,922), respectively. This patient was a female and manifested CPT in the left leg at birth.

In NF1-CPT, in addition to the identified germline heterozygous *NF1* inactivation, two patients (P14 and P15) were detected as carriers of a new *NF1* mosaic variant in their PA periosteum. Among them, c.989C > A (p.Ala330Glu) in P14 had an allele fraction of 34%, which was not found in her oral epithelial cells. According to ACMG criteria, this variant was classified as likely pathogenic (PM1 PM2 PM5 PP2 PP3) and was recorded in ClinVar as variant of uncertain significance (VUS). Another variant c.1381C > T (p.Arg461Ter) in P15 had an allele fraction of 18% in the PA tissue but not in the oral epithelial cells. This variant was recorded as pathogenic in ClinVar. Both patients were female, and they manifested CPT near 2 years old (24 months old for P14 and 22 months old for P15).

Non-NF1-CPT patients with somatic *NF1* inactivation have early and unilateral onset and similar union time to NF1-CPT

All nine non-NF1-CPT patients had unilateral onset with no family history of NF1 (Table 1). They all initially presented tibia bowing or fracture in infancy, including four infants presented at birth. The average age of onset was three months (Supp. Table S1). There was no significant bias in left or right lateral onset (5:4), but there were more male patients than females (6:3) in this cohort. H&E staining of the periosteum prevalently showed fibro-vascular tissue hyperplasia with partially hyaline degeneration, and thick-walled angiogenesis in non-NF1-CPT patients like NF1-CPT (Supp. Table S1). Compared with the six patients with NF1, the average age of tibia bowing or fracture presentation was two months, and the ratio of left to right lateralization was 2:4. Patients P11, P12, and P13 with bi-allelic *NF1* inactivation in PA tissue presented tibia bowing in three months after birth. Referring to the 34 non-NF1-CPT patients without somatic NF1 inactivation, the average age of tibia bowing or fracture presentation was near nine months, which was older than the nine non-NF1-CPT patients with somatic NF1 inactivation.

As for the union time of tibia after surgery, the average union time of the nine non-NF1-CPT patients with somatic *NF1* inactivation was five months. All the 43 non-NF1-CPT patients have a similar average union time (Supp. Table S1). Excluding the two patients (P11 and P13) without tibia union, the average union time of the other four NF1-CPT patients was four months. The union time of the NF1-CPT and non-NF1 CPT was quite close.

66.7% of NF1-CPT patients showed somatic *NF1* inactivation in PA tissue

Based on a review of 10 articles from 2006 to 2020 (Brekelmans et al. 2019; Lee et al. 2012; Leskela et al. 2009; Margraf et al. 2017; Paria et al. 2014; Sakamoto et al. 2007; Sant et al. 2015; Stevenson et al. 2006; Tahaei et al. 2018), a total of 57 patients with NF1-CPT were reported with *NF1* genetic testing in the PA tissue (Table 2). These patients were all diagnosed with NF1 based on the NIH diagnostic criteria. DNA, RNA, or protein from the PA tissue was detected using traditional molecular or cytogenetic detection methods (e.g., genotyping *NF1* markers, restriction fragment length polymorphism, MS-PCR, MLPA), and NGS-based technology (e.g., WES, RNA-seq). Thirty-seven patients were identified as having a second hit in *NF1* in the PA tissue other than a heterozygous germline *NF1* inactivation. Combined with the five of six NF1 patients in our study, a total of 42 NF1-CPT patients (42/63, 66.7%) were found to have a somatic *NF1* inactivation in addition to a germline

Table 2 Characteristics of genetic testing of NF1 on the PA tissue of CPT patients in literature

Year reported	1st Author	Patients (*n*)	Type of CPT	Method	With somatic NF1 second-hit?
2006	Stevenson et al.	2	NF1	Immuno-histochemical, genotyping 4 *NF1* markers	Yes
2007	Sakamoto et al.	1	NF1	RFLP, MS-PCR	No
2009	Leskela et al.	3	3 NF1 (with 3 controls without CPT and NF1)	*NF1* mRNA, protein	No
2012	Lee et al.	16	NF1	4 *NF1* markers, clonality assay	4 yes, 11 no
2014	Paria et al.	16	NF1	WES, RNA-seq, SNP microarray	12 *NF1* mutations, 4 no
2015	Sant et al.	1	NF1	WES	Yes in soft proliferative tissue, but not in cortical bone
2017	Margraf et al.	4	NF1	WES	3 yes
2018	Tahaei et al.	1	NF1	Single-cell RNA-seq	yes
2019	Brekelmans et al.	13	NF1	DNA + RNA (PTT, heteroduplex, FISH, southern Blot, Cytogenetic), MLPA, HaploPlex noonan syndrome kit	11 yes
2021	This study	6	NF1	WES + CNV	5 yes
Total		63			66.70%

mutation in the PA tissue, which formed bi-allelic *NF1* inactivation (Table 2).

No pathogenic variants were found in the known skeletal dysplasia causing genes

For differential diagnostic purposes, in the variants called from WES + CNV sequencing of PA tissue, we screened the non-benign variants of the prioritized genes related to skeletal dysplasia with features similar to those of CPT. The known phenotype-relevant genes include *GNAS* related to fibrous dysplasia, *AKT1*-associated Proteus syndrome, *PDGFRB,* and *NOTCH3*-related autosomal-dominant infantile myofibromatosis. Based on the ACMG criteria, no likely pathogenic variants of these genes were identified in the nine non-NF1-CPT and six NF1-CPT patients with *NF1* mutation. For the other 34 patients, only three missense variants with uncertain significance (VUS) were identified in the periosteum: P34 had c.166_167delGCinsAT (p.Ala56Met) in *GNAS*, P36 had c.881C > T (p.Thr294Met) in *NOTCH3,* and P47 had c.914C > T (p.Thr305Ile) in *AKT1*.

Discussion

This study focused on non-NF1-CPT patients that did not meet the clinical diagnostic criteria for NF1. These patients had an isolated clinical manifestation of tibial pseudarthrosis, and we primarily detected the PA periosteum using WES + CNV analysis to screen for disease-causing variants. We found that 21% of the detected non-NF1-CPT patients had somatic mono-allelic *NF1* inactivation in the PA periosteum but not in blood or oral samples. Furthermore, using the same pipeline in the six NF1-CPT patients, we identified a second somatic *NF1* inactivation in addition to germline mono-allelic *NF1* inactivation in the PA periosteum from five of them. Our findings revealed that somatic *NF1* variation exists in non-NF1-CPT and NF1-CPT patients with a significant proportion, and both have early onset.

NF1 somatic mutation found in the pathological periosteum of non-NF1-CPT with fibrous proliferation could be construed as low-level mosaicism of NF1. The age of tibia bowing or fracture onset, tibia union time after surgery, and fibrosis in the periosteum had no significant differences between the non-NF1-CPT patients with *NF1* somatic mutation and the NF1-CPT patients. NF1 has high phenotypic variability without clear genotype–phenotype correlations (Gutmann et al. 2017; Sabbagh et al. 2013; Zhang et al. 2015). The function of neuro-fibromin encoded by *NF1* is still not fully understood. Recently, Luo et al. and Koliou et al. reported that neuro-fibromin is involved in chromosome congression and participates in somatic cell division (Koliou et al. 2016; Luo et al. 2014). This may be related to the various onset times of different NF1 manifestations. Similar to the most common CALs, CPT as a skeletal abnormality is usually symptomatic from birth to infancy (Gutmann et al. 2017). In this study, non-NF1-CPT patients did not exhibit CALs and other symptomatic manifestations, the localized lesion in tibia with *NF1* somatic haplo-insufficiency during bone development could be an importance cause. Furthermore, as periosteum serves as the source of osteoprogenitor cells producing chondrocytes and mature

osteoblasts for bone growth, bone modeling and remodeling after fracture (Maes et al. 2010; Ozaki et al. 2000; Roberts et al. 2015), *NF1* inactivation was found in the considerable portion of CPT's PA periosteum with highly fibrous hyperplasia might contribute to the formation of pseudarthrosis. The dysfunction of periosteum could also impair the ossification during bone growth and bone-healing after fracture.

A high frequency of LoF variations of *NF1* was observed in CPT, either germline de novo or somatic. After combining the results of previous studies and our study, the overall proportion of somatic second hits of *NF1* in NF1-CPT is approximately 67% (Brekelmans et al. 2019; Lee et al. 2012; Leskela et al. 2009; Margraf et al. 2017; Paria et al. 2014; Sakamoto et al. 2007; Sant et al. 2015; Stevenson et al. 2006; Tahaei et al. 2018). Interestingly, this proportion is the same as that of the somatic inactivation in skin biopsy and blood mosaicism of *NF1* of the 15 MNF1 cases subjected to genetic testing (10/15, 67%) (Garcia-Romero et al. 2016). With 21 of 63 NF1-CPT cases harboring somatic second hits in the literature and nine of 43 non-NF1-CPT cases with somatic mosaicism in this study, the overall rate of mosaic somatic *NF1* inactivation was about 50% in 106 cases. This proportion is close to that of the de novo germline frequency of *NF1* inactivation (Evans et al. 2010; Zhu et al. 2019). However, the cause of such a high-variation frequency of NF1 is not fully understood.

With *NF1* pathogenic variants identified, non-NF1-CPT caused by somatic mosaicism and NF1-CPT caused by germline *NF1* inactivation will be benefit for precise treatment, follow-up management, and gene counseling in future. Molecular testing can help establish the diagnosis of NF1 and MNF1 for CPT as early. Recently Legius et al. revised the diagnostic criteria for NF1 by adding *NF1* genetic diagnosis and several more specified clinical features (Legius et al. 2021; Parrozzani et al. 2015; Tadini et al. 2014; Vagge et al. 2016). With a clear diagnosis, the follow-up surveillance of tibia union and other tissue manifestations between NF1-CPT and non-NF1-CPT would be helpful for future customized management. Furthermore, up to 13% of the reported patients with MNF1 or complete NF1 have malignancy risk (Garcia-Romero et al. 2016). Malignant peripheral nerve sheath tumors (MPNSTs) are the most common and often occur in adolescents or early adulthood in individuals with NF1 (Hagel et al. 2007; McCaughan et al. 2007; Valentin et al. 2016). Patients with complete *NF1* loss are at a greater risk of developing MPNST (Kehrer-Sawatzki et al. 2017; Upadhyaya et al. 2004) than other types of variants. In this study, Patient P1 was detected the entire *NF1* loss in the PA periosteum, and the follow-up surveillance for malignant risk would be needed. Other tumors, such as rhabdomyosarcomas and malignant fibrous histiocytoma, may occur since childhood (Seminog and Goldacre 2013; Varan et al. 2016). Although non-NF1-CPT with a somatic mutation has a lower risk of malignant tumors than NF1-CPT and unlikely has NF1 offspring, follow-up management would be better for individuals with *NF1* inactivation.

In addition to *NF1*, other genes might cause musculoskeletal dysplasia features that need to be differentiated from CPT. For example, polyostotic fibrous dysplasia is caused by an early embryonic postzygotic somatic mutation of *GNAS*. We screened *GNAS* in our tissue WES + CNV analysis, and no patient had pathogenic variants of this gene. Proteus syndrome is characterized by hamartomatous overgrowth of multiple tissues, such as hyperostosis, and the somatic mosaic of *AKT1* contributes to 90% of affected individuals. None of our CPT cases in this study were identified any pathogenic variants of *AKT1* in the detected tissue. The variant in P47 might be upgraded to likely pathogenic if it is de novo. As the parent's samples were unavailable, the pathogenicity of this variant would be illustrated later. We also screened *PDGFRB* and *NOTCH3* gene related to autosomal-dominant infantile myofibromatosis, and no pathogenic variants were identified. All cases in this study confirmed that there were no pathogenic or likely pathogenic variants in these genes. Excepting these four genes and *NF1*, other genes were identified in patients sporadically with likely pathogenic or LoF variants. Whether other genes participated in CPT etiology needs to be further investigated.

Due to available samples and testing technology limitations, 79% of the non-NF1-CPT cases were genetically negative and only the gDNA of patients were detected using WES + CNV plus MLPA. According to previous studies, approximately 1/3 of the variants affecting splicing could not be detected by gDNA sequencing (Evans et al. 2016) and cDNA sequencing demonstrated a relatively higher diagnostic sensitivity for *NF1* than solely gDNA-based analysis. The lack of cultured cells derived from fresh amorphous mesenchymal tissues prevented the assessment of mRNA expression. In addition, this study was lack of whole genome sequence analysis that did not enable identification of pathogenic *NF1* mutations that could alter expression of the gene or splicing of the mRNA leading to nonsense-mediated decay. Some structural variants such as cytogenetic rearrangements were not detected, which contributed to < 1% of NF1 affected individuals (Messiaen et al. 2000). Therefore, the rate of somatic mosaicism for non-NF1-CPT could be higher than previously reported. Further studies on the remaining NF1-testing negative CPT patients to investigate other disease-causing variants are required. Other limitations included the lack of matching normal periosteum for all 49 patients, and we were unable to do the pair-wise comparison of PA-affected tissues to receive a more substantial result. In addition, some patients lacked of long-term follow-up and thorough physical examination. Thus, the clinical features presented in the collected patients might have been incomplete by the time of documentation. Moreover, some clinical

manifestations are rare and of late-onset, such as subcutaneous neuro-fibromas, glioma, and MPNST, and they may be asymptomatic until adolescence or adulthood. Therefore, a complete follow-up, precise diagnosis, prognosis, or clinical course will be useful for non-NF1-CPT and NF1-CPT.

Conclusion

We found a significant proportion of non-NF1-CPT patients without NF1 diagnosis but with somatic mono-allelic *NF1* inactivation in the PA periosteum. The high frequency of *NF1* somatic mutations occurred in both non-NF1-CPT and NF1-CPT patients at an early age. During bone development and growth, *NF1* haplo-insufficiency in the PA periosteum may contribute to the over proliferation of fibrous tissue leading to the formation of CPT.

WEB resources used

UCSC: https://genome.ucsc.edu/
OMIM: https://www.omim.org/
wInterVar: https://wintervar.wglab.org/
Varsome: https://varsome.com/
GnomAD: https://gnomad.broadinstitute.org/
HGMD: http://www.hgmd.cf.ac.uk/ac/validate.php
ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/
Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

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Availability of data and materials The novel variants found in this study were submitted to ClinVar (accession: SCV002014754-SCV002014759). Other datasets used during the current study are available from the corresponding authors on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval and consent to participate This study was approved by the Ethics Committee of Hunan Children's Hospital (Approval No. HCHLL-2020-106). The samples were obtained appropriate informed consent from all participants.

Consent for publication All authors read and approved the final manuscript for publication.

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