

ORIGINAL ARTICLE

Absent expression of the osteoblast-specific maternally imprinted genes, *DLX5* and *DLX6*, causes split hand/split foot malformation type 1

Sawitree Rattanasopha,¹ Siraprapa Tongkobpetch,^{2,3} Chalurmporn Srichomthong,^{2,3} Pravit Kitidumrongsook,⁴ Kanya Suphapeetiporn,^{2,3} Vorasuk Shotelersuk^{2,3}

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/jmedgenet-2014-102576>).

¹Doctor of Philosophy Program in Medical Sciences, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

²Department of Pediatrics, Center of Excellence for Medical Genetics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

³Excellence Center for Medical Genetics, King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Bangkok, Thailand

⁴Department of Orthopedics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Correspondence to

Dr Kanya Suphapeetiporn, Division of Medical Genetics and Metabolism, Department of Pediatrics, Sor Kor Building 11th floor, King Chulalongkorn Memorial Hospital, Bangkok 10330, Thailand; kanya.su@chula.ac.th

Received 12 June 2014
 Revised 15 September 2014
 Accepted 30 September 2014

ABSTRACT

Background Split hand/split foot malformation (SHFM) type 1 is characterised by missing central digital rays with clefts of the hands and/or feet, which was linked to chromosome 7q21.3. While double knockout of *Dlx5* and *Dlx6* resulted in limb defects in mice, the majority of patients with SHFM1 had only heterozygous chromosomal abnormalities.

Objective To investigate the clinical and molecular features of a large family with SHFM1.

Methods Blood samples of family members were investigated by linkage analysis, array comparative genomic hybridisation, exome sequencing and PCR-Sanger sequencing. Cultures from bone specimens obtained from the proband and an unrelated unaffected individual were established and subjected to quantitative real-time PCR, reverse-transcribed PCR, Western blot and imprinting analysis.

Results We report a large pedigree of SHFM1 with 10 members having a heterozygous 103 kb deletion, the smallest one ever reported to be associated with SHFM1. Of these 10, two had no limb anomalies, making a penetrance of 80%. The deletion encompassed exons 15 and 17 of *DYNC111*, which are known enhancers of two downstream genes, *DLX5* and *DLX6*. Surprisingly, *DLX5* and *DLX6* RNA and proteins in our proband's cultured osteoblasts, instead of 50% decrease, were absent. Allelic expression studies in cultured osteoblasts of the unaffected individual showed that *DSS1*, *DLX6* and *DLX5* expressed only paternal alleles. These lines of evidence indicate that *DSS1*, *DLX6* and *DLX5* were maternally imprinted in osteoblasts.

Conclusions SHFM1 in our family is caused by a heterozygous paternal deletion of enhancers of the osteoblast-specific maternally imprinted *DLX6* and *DLX5* genes, leading to the absence of their proteins.

INTRODUCTION

Split hand/split foot malformation (SHFM) is one of the most complex limb abnormalities, characterised by missing central (second, third and/or fourth) digital rays with clefts of the hands and/or feet and fusion of remaining digits.¹ The prevalence of SHFM has been reported to be 0.60/10 000 in Caucasians.² SHFM can be presented in either an isolated form or in combination with additional anomalies.³

SHFM disorders are genetically heterogeneous with multiple loci and different inheritance patterns. For the isolated form, nine different genetic

loci have been mapped and two genes have been identified: SHFM1 (MIM 183600) at chromosome 7q21.3, SHFM2 (MIM 313350) at Xq26, SHFM3 (MIM 246560) at 10q24, SHFM4 (MIM605289) due to mutations in *P63* at 3q27, SHFM5 (MIM606708) at 2q31 and SHFM6 (MIM225300) caused by mutations in *WNT10B* at 12q13. In addition, three more loci on 8q21.1–q22.3,⁴ 4q32–4q35⁵ and 6q16–6q22⁵ were reported to link to SHFM.

The 7q21.3 region associated with SHFM1 includes the candidate genes *DSS1*, *DLX5* and *DLX6*, which are important in limb development.^{6,7} Knocking out both alleles of either *Dlx5* or *Dlx6* was lethal in mice and showed craniofacial defects, but did not lead to limb abnormalities. To manifest SHFM phenotype, both alleles of *Dlx5* and *Dlx6* were needed to be knocked out.^{8,9} In humans, the majority of patients with SHFM1 were heterozygous for chromosomal structural abnormalities. Only two families with limb defects were found to have point mutations in *DLX5*. The first was a consanguineous family with two siblings having SHFM and hearing loss. Both were found to have a homozygous missense mutation in *DLX5*, c.A533C (p.Q178P).¹⁰ The second family had two affected members, a mother and her son, with split feet and triphalangeal thumbs. Both had a heterozygous missense mutation in *DLX5*, c.G558T (p.Q186H).¹¹ All other patients associated with the SHFM1 locus were caused by deletions or complex chromosomal rearrangements.

Studies in both mice and zebrafish have identified novel tissue-specific enhancers located within the coding regions of *Dync1i1* (dynein, cytoplasmic 1, intermediate chain 1) that regulate *Dlx5* and/or *Dlx6*.⁷ A recent study identifying a 106 kb deletion encompassing exons 11–18 of the *SLC25A13* gene and exons 14–17 of the *DYNC111* gene in patients with SHFM1 has confirmed a key role of *DYNC111* exonic enhancers in normal limb formation in humans.¹²

Here, we describe a large pedigree with SHFM1. A heterozygous 103 kb deletion, the smallest ever reported, encompassing parts of *DYNC111* and *SLC25A13*, was identified. Surprisingly, *DLX5* and *DLX6* proteins in our proband's cultured osteoblasts were absent. Allelic expression studies in cultured osteoblasts of an unaffected individual showed that *DSS1*, *DLX6* and *DLX5* were maternally imprinted in osteoblasts. These findings

To cite: Rattanasopha S, Tongkobpetch S, Srichomthong C, et al. *J Med Genet* Published Online First: [please include Day Month Year] doi:10.1136/jmedgenet-2014-102576

Developmental defects

indicate that SHFM1 in our family is caused by a heterozygous paternal deletion of enhancers of the osteoblast-specific maternally imprinted *DLX6* and *DLX5* genes, leading to the absence of their proteins.

PATIENTS AND METHODS

Linkage analysis

After written informed consent was obtained, genomic DNA was extracted from peripheral blood leucocytes using commercially available kits (Qiagen, Valencia, California, USA). We performed linkage analysis in 14 family members by genotyping microsatellite markers at eight autosomal SHFM loci. This included SHFM1 on chromosome 7q21 (D7S657 and D7S515), SHFM3 on chromosome 10q24 (D10S192 and D10S597), SHFM4 on chromosome 3q28 (D3S1601 and D3S1580), SHFM5 on chromosome 2q31 (D2S335 and D2S364), SHFM6 on chromosome 12q13 (D12S368) and loci on chromosome 4q32–4q35 (D4S413, D4S1597 and D4S1539), 6q16–6q22 (D6S434, D6S287 and D6S262) and 8q21.11–q22.3 (D8S270 and D8S1784). The details of the primers were obtained from Marshfieldmap (<http://research.marshfieldclinic.org/genetics/home/index.asp>). We typed all fluorescently labelled PCR products on an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, California, USA) with GeneMapper software (Applied Biosystems). The MLINK program (available from <http://linkagerockefeller.edu/rockefeller.edu/>) was used to calculate the two-point linkage analysis with the model of autosomal-dominant inheritance with a high penetrance.

Copy number analysis by array comparative genomic hybridisation

Genomic DNA of the proband and a normal Thai control were sent to MacroGen (Seoul, South Korea) to determine copy number variations. DNA was independently labelled with fluorescent dyes, co-hybridised to specific sequences on chromosome 7 using NimbleGen 385K chip (cat. no. B3738001-00-01; Roche NimbleGen Systems, Madison, Wisconsin, USA) and scanned using a 2 µm scanner. Log₂-ratio values of the probe signal intensities (Cy3/Cy5) were calculated and plotted versus genomic position using Roche NimbleGen NimbleScan software. Data were displayed in Roche NimbleGenSignalMap software.

Breakpoint identification

To define the breakpoints, we performed PCR with multiple pairs of primers flanking the deletion including FLANK-F: 5'-CCGCCATACAACCTCATTT-3' and FLANK-R: 5'-GATGTCTGAAGTCAGCAACC-3', followed by Sanger sequencing.

Segregation analysis and screening for the deletion in ethnic matched controls

To determine SHFM1 genotypes in all 19 available family members, we used a duplex PCR strategy. Two pairs of primers, FLANK-F and FLANK-R to amplify the 2245 bp deleted allele on chromosome 7q21 and 5'-CTTGTGTACTTTGGCTTTCATTAC-3' and 5'-GCTCTATATTTACTGCAGCACAGAAC-3' to amplify a 1188 bp control allele of *KMT2C*, were used for PCR amplification. PCR products were resolved on a 1.2% agarose gel. This pair of primers was also used to analyse 150 unrelated unaffected Thai controls.

Determination of point mutations in candidate genes

PCR amplification of all coding regions of the *DYNC111*, *DSS1*, *DLX6* and *DLX5* genes was carried out using primers and annealing temperatures in online supplementary table S1. PCR

products were treated with ExoSAP-IT (USP Corporation, Cleveland, Ohio, USA) and sent for direct sequencing at MacroGen. Sequence data were analysed using Sequencher (V5.0; Gene Codes Corporation, Ann Arbor, Michigan, USA).

Exome sequencing

Exome sequencing using high-quality genomic DNA from peripheral blood of the proband (IV-30) was performed by MacroGen. DNA was captured on the TruSeqExome Enrichment Kit (Illumina) and subsequently sequenced on the HiSeq2000 instrument. Sequence reads were mapped against UCSC hg19 using BWA software (<http://bio-bwa.sourceforge.net/>). The SNPs and Indels were detected by SAMTOOLS (<http://samtools.sourceforge.net/>) and annotated by dbSNP&1000G. Then we sought for pathogenic mutations in the 26 candidate genes in the 9 SHFM loci, including *BHLHA9*, *BTRC*, *CHD3*, *DACTYLIN*, *DLX1*, *DLX2*, *DLX5*, *DLX6*, *EVX2*, *EPS15L1*, *FGF8*, *FGF13*, *HOXD2*, *HOXD5*, *HOXD6*, *HOXD7*, *HOXD9*, *HOXD10*, *HOXD11*, *HOXD12*, *HOXD13*, *LBX1*, *PBXW4*, *POLL*, *TP63* and *WNT10B*.^{3 13}

Human bone culture

Bone tissues were taken from the proband (IV-30) during an operation to close the cleft on his right hand. We obtained a bone chip including cortical and cancellous bones from his right third metacarpal. A bone chip of an unrelated unaffected individual undergoing surgery after an accident was also obtained. Cells were seeded in a T-25 cell culture flask, cultured in DMF-12 (Gibco) supplemented with 20% fetal bovine serum, 1% penicillin–streptomycin and incubated in a humidified atmosphere with 5% CO₂ at 37°C. Passages 3–5 were used for experiments. Osteoblast differentiation was induced by the addition of 50 µg/mL ascorbic acid and 10 nM dexamethasone.

RNA expression of cultured osteoblasts

After three passages, total RNA was extracted from bone cultures using a QIAamp RNA blood mini kit (Qiagen). Reverse transcription was performed using ImProm-IITM RT (Promega, Madison, Wisconsin, USA). RNA levels of *DYNC111*, *DSS1*, *DLX6* and *DLX5* were quantified by quantitative real-time (qRT) analysis using TaqMan probes of the StepOnePlus Real-time PCR system (Applied Biosystems) (see online supplementary table S2). For *DYNC111*, we performed qRT using two pairs of primers; one for amplifying exons 11–12, 5' to the deletion, and the other for amplifying exons 16 and 17, which were within the deletion. The probes were run in triplicate two times in separate tubes. Relative expression analysis was calculated in terms of ΔΔCt normalised to *GAPDH* transcription levels. We also determined the expression ratio of the *DYNC111*, *DSS1*, *DLX6* and *DLX5* in each bone culture sample by PCR using complementary DNA (cDNA) as template. The PCR products were separated on a 2% agarose gel to analyse gene transcripts. Sequences of primers are available in online supplementary table S3.

Protein expression of cultured osteoblasts

The antibodies including a monoclonal mouse anti-DYNC111 (catalogue #ab23905, Abcam, Cambridge, UK), a monoclonal rabbit anti-DLX6 (catalogue #ab137079, Abcam), a polyclonal rabbit anti-DLX5 (catalogue #ab109737, Abcam), a monoclonal rabbit anti-alpha tubulin (catalogue #ab52866, Abcam) and a polyclonal rabbit anti-DSS1 (catalogue #1, Proteintech, Chicago, Illinois, USA) were obtained.

Protein was extracted from bone cultures using ice-cold RIPA lysis buffer with Halt protease inhibitor cocktail (Pierce,

Rockford, Illinois, USA) and protease inhibitor cocktail (Sigma Aldrich, Sigma, Missouri, USA). The total soluble protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce). Sodium dodecyl sulfate (SDS)-containing buffer was added to supernatant and the sample was denatured by heating for 7 min before SDS-polyacrylamide gel electrophoresis (PAGE) loading. After size separation by 4%–15% SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes by iBot Gel Transfer System (Invitrogen).

The membrane was stripped and re probed with 1:1000 anti- α tubulin (Abcam) and 1:1500 goat anti-rabbit IgG HRP: sc-2030 (Santa Cruz Biotechnology, Santa Cruz, California, USA).

Imprinting analysis

We obtained a blood sample and a bone chip from the right femur of an unaffected individual during an operation for his fracture. Blood samples from his parents were also obtained. Genomic DNA was extracted from their blood samples. Osteoblasts were cultured from the bone chip. RNA was extracted from the cultured osteoblasts and reversed transcribed to cDNA. PCR using genomic DNA of this individual followed by direct sequencing of the *DSS1*, *DLX5* and *DLX6* entire coding, 5'UTR and 3'UTR regions was performed.

SNPs were identified within exons of *DSS1*, *DLX6* and *DLX5* using the National Center for Biotechnology Information SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). Primer sequences used for allele-specific expression analyses for *DSS1*, *DLX6* and *DLX5* are available in online supplementary table S3. We incubated the total RNA with or without RT, including an oligo (dT)₁₅ primer to confirm that no PCR amplification signals could be detected in the absence of RT. The regions of the genomic DNA and cDNA containing SNPs were PCR-amplified using HotStarTaq DNA polymerase (Qiagen) and primers (see online supplementary table S3). The PCR products were subjected to Sanger sequencing. Allelic expression was determined by comparing sequences of the child's cDNA and his parents' genomic DNA.

RESULTS

Clinical studies

We investigated 19 members from a large Thai family including 8 affected and 11 unaffected with SHFM (figure 2A, see online supplementary figure S1). None reported fractures or had ear abnormalities. Physical examination revealed that the spectrum of clinical manifestations was broad (figure 1, see online supplementary table S1 and figure S2), ranging from the presence of cutaneous syndactyly between the second and third digits of right hand as in III-4 (see online supplementary figure S2) to bilateral split hands and feet in II-11 (figure 1).

Linkage analysis

We first performed linkage analysis in 14 individuals on eight known autosomal loci (SHFM1, SHFM3-6, 4q32-4q35, 6q16-6q22 and 8q21.11-q22.3). We were able to exclude linkage to all loci except the SHFM1 locus on chromosome 7q21. Using markers D7S657 and D7S515, the maximum two-point logarithm of odds score was 2.41 at zero recombination (figure 2A).

Copy number analysis and breakpoint identification

Using a targeted 385K chromosome 7-specific microarray, we identified an approximately 100 kb deletion on 7q21 (figure 3A). Sanger sequencing of PCR products spanning the breakpoints revealed a 103 763 bp deletion, g.95694099_95797866delins TCATC (figure 3B), encompassing exons 14–17 of the *DYNC111* gene and exons 13–18 of the *SLC25A13* gene. The deletion did not encompass an otic vesicle enhancer that was found to be located in *SLC25A*.

Segregation analysis and screening for the deletion in ethnic matched controls

All 19 available family members were genotyped. We found that the deletion was present in all eight patients and in two unaffected individuals (III-30 and IV-9) (figure 2B), but absent in 150 unaffected control individuals (300 chromosomes).

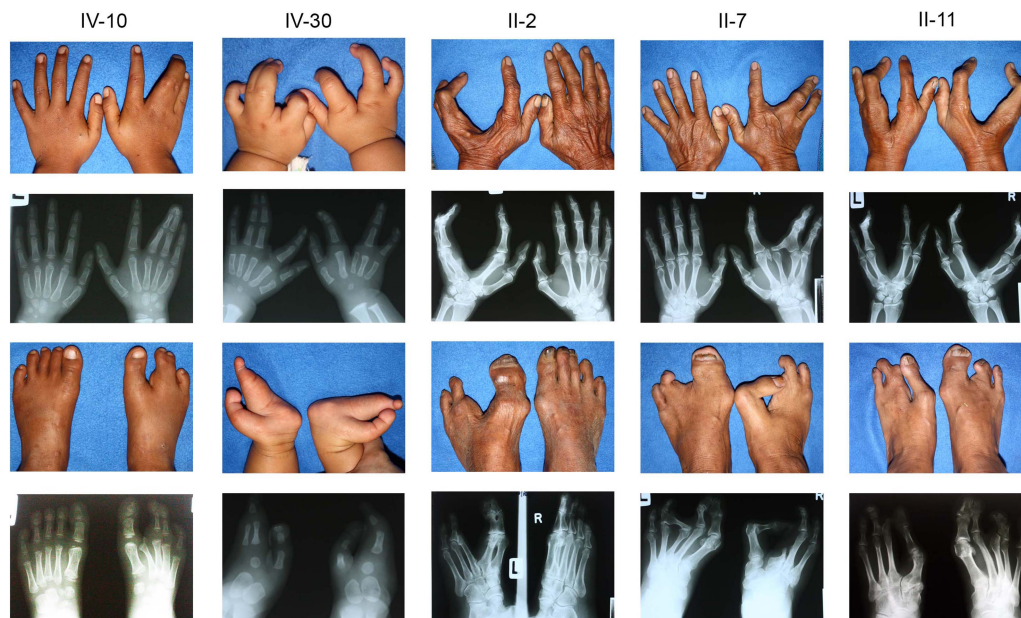


Figure 1 Clinical and radiological features of hands and feet of patients.

Developmental defects

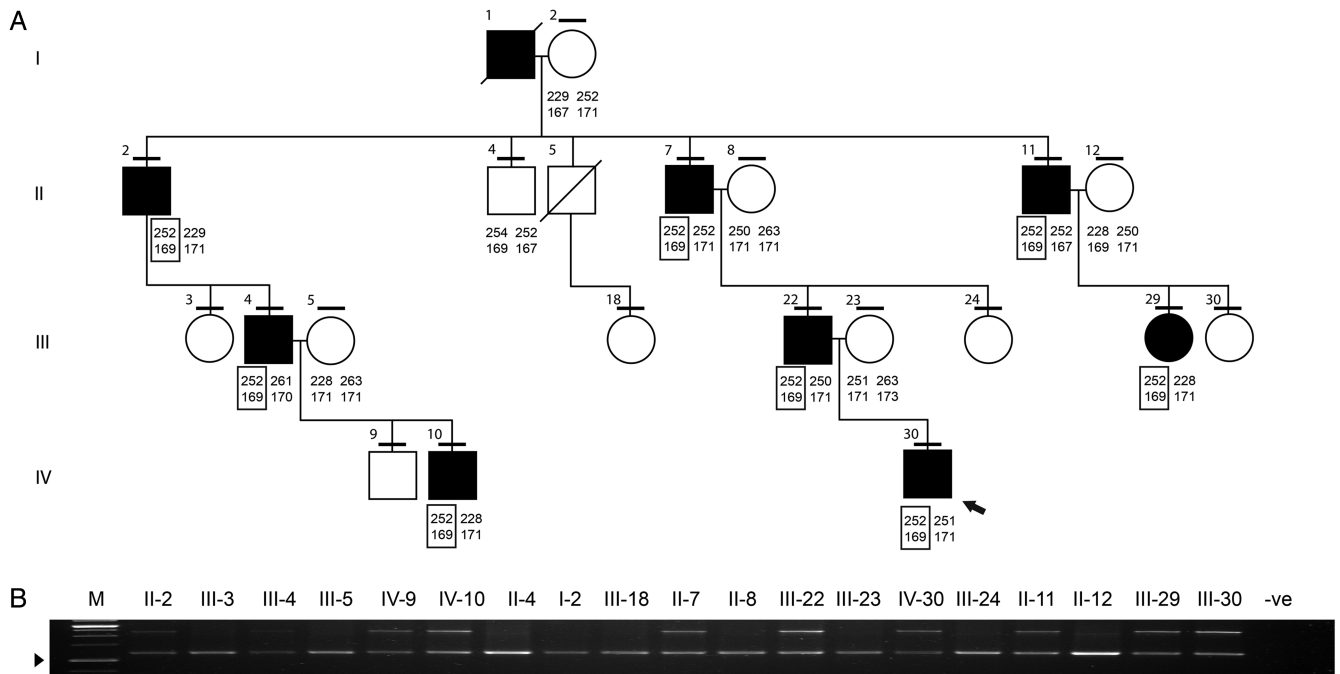


Figure 2 (A) Pedigree of the family and genotype of microsatellite markers in the region of split hand/split foot malformation-1 on chromosome 7q21.3. Affected individuals are represented by filled symbols and unaffected individuals by open symbols. The boxes defined by microsatellite markers D7S657 and D7S515 cosegregate with the disease. (B) PCR amplification of the 103 kb deletion allele and *KMT2C*, used as a control gene. Lane 1 contains a DNA size marker (M) with an arrow head indicating a size of 1000 bp; lanes 2–19 represent the respective family members. The upper bands present in II-2, III-4, IV-9, IV-10, II-7, III-22, IV-30, II-11, III-29 and III-30 are the amplified products of the deletion allele (2245 bp). The lower 1188 bp bands present in all family members are a part of the *KMT2C* gene, used as a PCR control.

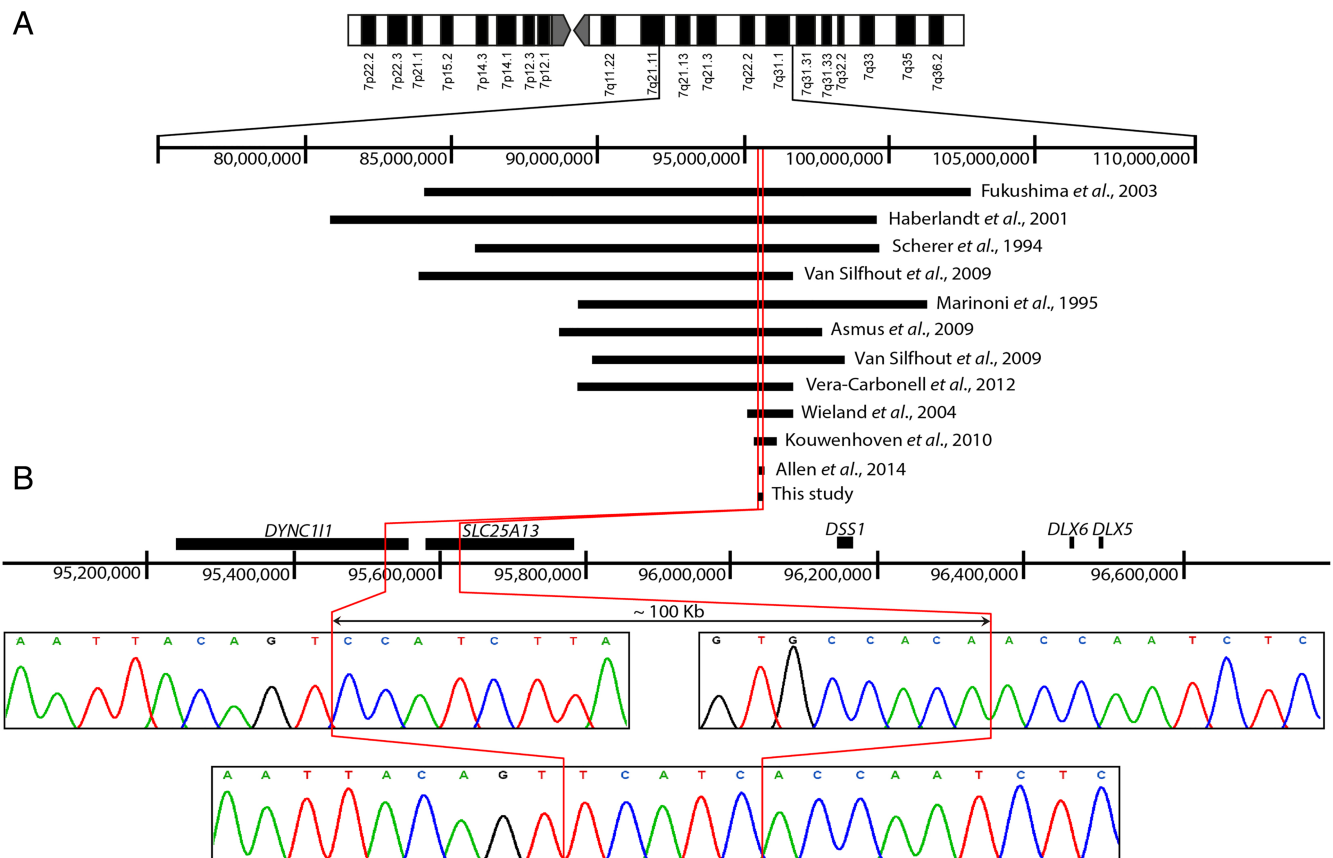


Figure 3 (A) Previously reported chromosome 7 deletions in split hand/split foot malformation-1 and (B) sequencing chromatogram of the breakpoints of our proband showing the distal and proximal breakpoints with a 5 bp insertion.

Exclusion of point mutations

To be certain that no point mutations in the coding regions of *DYNC111*, *DSS1*, *DLX6* and *DLX5* contributed to or modified the phenotype, we PCR-sequenced the entire coding regions of these four genes in the proband and found no mutations.

Exome sequencing

To exclude other pathogenic mutations in genes within the 9 linked loci of SHFM that might affect the phenotype of our proband, exome sequencing was performed.

Exome sequencing generated 5.83 Gb of sequence data, with 71.4% of target regions covered at least 10 times. No pathogenic mutations in the 26 genes linked to the SHFM loci were identified.

Osteoblast culture and determination of *DLX5* and *DLX6* expression levels

As enhancers of *DLX5* and *DLX6* reside in exons 15 and 17 of *DYNC111*, we hypothesised that the deletion encompassing exons 14–17 of *DYNC111* could alter the expression of nearby genes leading to the SHFM phenotype. We used qRT-PCR, PCR using cDNA as template and Western blot analysis to determine RNA and protein levels of *DYNC111*, *DSS1*, *DLX5* and *DLX6* in the proband's cultured osteoblasts. The RNA level of

DYNC111 using primers amplifying exons 11–12, which were 5' to the deletion, was 46% of the control. This was statistically significantly higher than 32% of the control when primers amplifying exons 16–17, which were within the deletion, were used (see online supplementary figure S3). As expected, the protein levels of *DYNC111* were approximately half compared with the control. RNA and protein levels of *DSS1* were similar to those of controls (figure 4). Surprisingly, RNA and protein of *DLX5* and *DLX6* were absent (figure 4).

Imprinting analysis

Since the *DLX5* and *DLX6* of the proband who harboured a heterozygous paternal deletion were not expressed, we hypothesised that both were maternally imprinted genes. We studied the imprinting phenomenon of these genes in osteoblasts of an unrelated unaffected individual. We first identified heterozygous variants whose parental origin could be determined in *DSS1*, *DLX5* and *DLX6*. At the *DSS1* 7:96317968 position (hg 19), the individual, his father and his mother were CT, CC and CT, respectively. At the *DLX5* rs 73708843, the individual, his father and his mother were CT, CC and CT, respectively. At the *DLX6* rs 2272280, the individual, his father and his mother were CT, TT and CC, respectively.

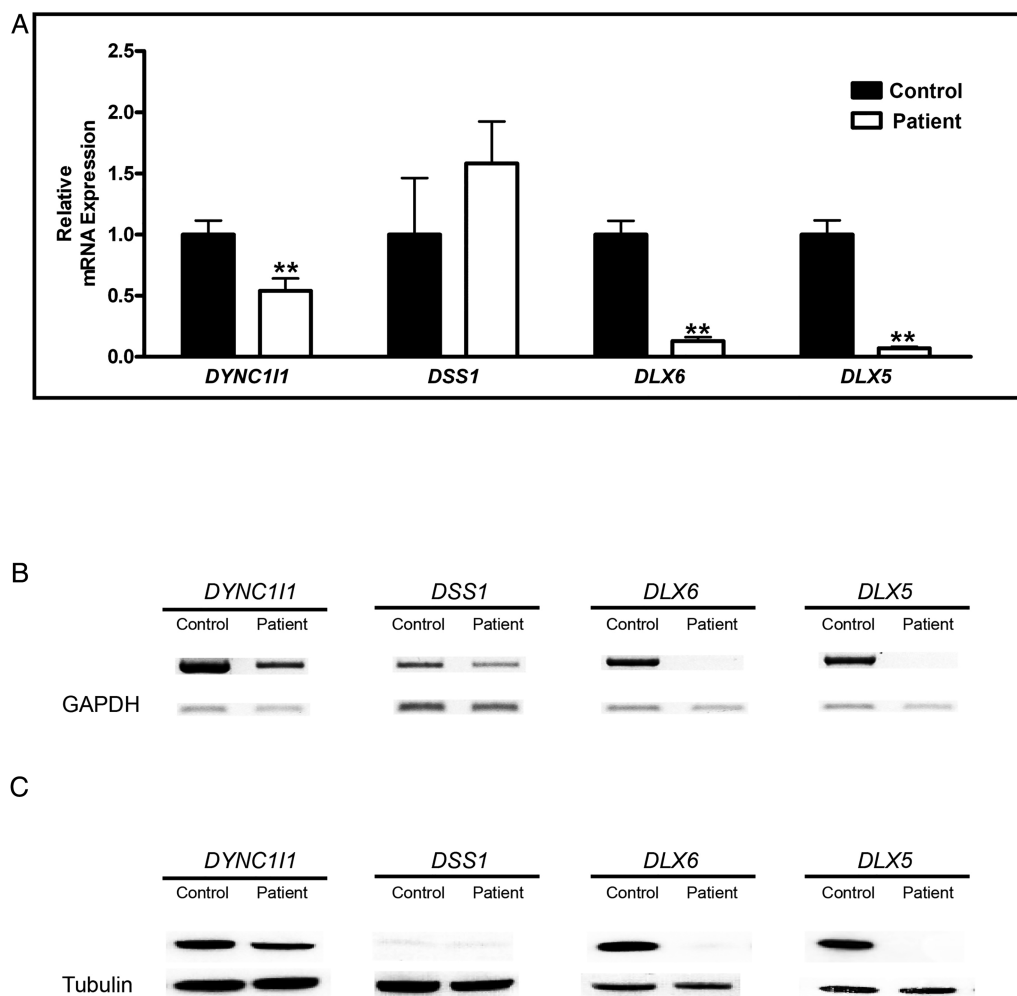


Figure 4 Expression of *DYNC111*, *DSS1*, *DLX6* and *DLX5* in cultured osteoblasts of our proband and an unaffected individual. (A) RT-PCR analysis: graphical representation of the relative RNA expression. (B) Semi-quantitative assay. Total RNA was used for cDNA synthesis; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (C) Western blot analysis.

Developmental defects

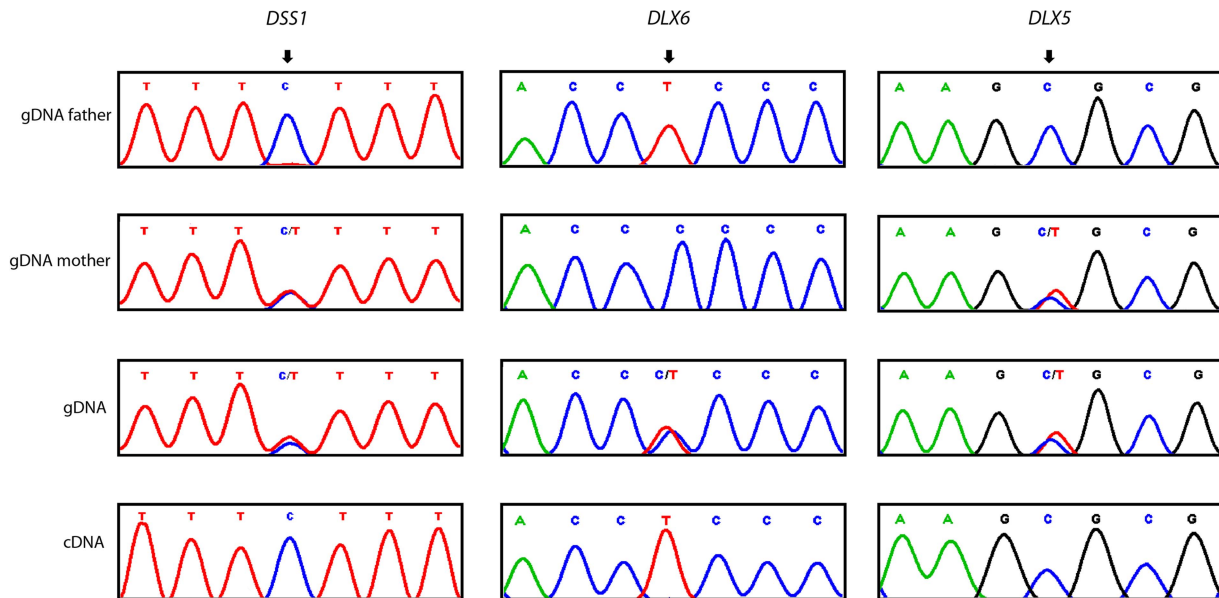


Figure 5 Imprinting analysis of the *DSS1*, *DLX6* and *DLX5* genes in cultured osteoblasts of an unaffected individual. The three upper panels used genomic DNA of leucocytes as template for PCR, while the lowest panel used complementary DNA, reverse-transcribed from RNA extracted from an unaffected individual's cultured osteoblasts.

We then PCR-sequenced this unaffected individual's cDNA from cultured osteoblasts, which showed that only the C allele of *DSS1*, the C allele of *DLX5* and the T allele of *DLX6* were expressed. All were paternal alleles (figure 5).

DISCUSSION

We identified a large Thai pedigree with 10 members having a 103 763 bp deletion encompassing the exons 15 and 17 of *DYNC111*. The trait exhibited a great range of severity. Of these 10, two members (III-30 and IV-9) had absolutely no abnormalities, making a penetrance rate of 80%. Among the remaining eight members who exhibited limb abnormalities, the severity ranged from mild cutaneous unilateral syndactyly of the second and third digits (III-4), to severe involvement of both hands and feet (II-11). Besides non-penetrance and variable expressivity, we observed an unusual phenotype. The proband's father (III-22) showed a central polydactyly of his right hand (see online supplementary figure S2). Unfortunately, we do not have pictures or radiographs of his right hand before surgery to remove the extra digit. No polydactyly has previously been reported in SHFM1.

We then performed experiments to determine the molecular pathology in our family with SHFM. Because eight autosomal loci have been linked to SHFM, we started with linkage analysis using 2–3 microsatellite markers for each locus. Seven loci were excluded, leaving only the SHFM1 locus. We subsequently went on to examine whether there was a deletion in this locus. Using array comparative genomic hybridisation followed by PCR and direct sequencing, we were able to identify the deletion and its breakpoints causing SHFM in this family. Previously the SHFM1 critical region was around 888 000 bp¹⁴ and very recently was narrowed down to 105 935 bp.¹² Here, we report the smallest 103 763 bp deletion encompassing exons 14–17 of the *DYNC111* gene and exons 13–18 of the *SLC25A13* gene. The deletion was found in all eight affected and two unaffected members. PCR and direct sequencing of the entire coding regions of the *DYNC111*, *DSS1*, *DLX6* and *DLX5* genes showed no mutations. Exome sequencing revealed no mutations of

genes in the linked SHFM loci. We, therefore, concluded that the molecular pathology, causing SHFM in our family, was this 103 763 bp deletion.

Since *DYNC111* is not expressed in limbs during embryogenesis¹⁵ and homozygous *SLC25A13* mutations cause citrin deficiency (MIM 603859),¹⁶ both genes are unlikely to be responsible for SHFM1. As previously reported in zebrafish, mice and humans, the exons 15 and 17 of *DYNC111* also act as enhancers of *DLX5/6* expression.^{7 17} Both genes express in limbs during mouse embryonic day 12.5.^{8 9 18 19} We hypothesised that haploinsufficiency of *DLX5* and *DLX6* caused SHFM1. After obtaining a bone chip of the proband, we cultured osteoblasts and determined expressions of *DYNC111*, *DSS1*, *DLX5* and *DLX6*. RNA and protein levels of *DYNC111* were approximately half of the control. Although *DSS1* protein was technically difficult to detect,²⁰ we showed that RNA and protein levels of *DSS1* were similar to those of the control. Surprisingly, RNA and protein levels of *DLX5* and *DLX6* of the proband's cultured osteoblasts were absent, instead of being half of those of the control. This is consistent with findings in knockout mouse experiments. Only homozygous *dlx5/dlx6* double-knockout mice showed a split hand/foot type I phenotype, while heterozygous *dlx5/dlx6* double-knockout mice showed no obvious limb defects.^{8 9}

Since only one allele was deleted but no protein was present, we hypothesised that *DLX5* and *DLX6* were imprinted genes. Moreover, the pedigree revealed that all patients inherited the deleted allele from their father. Thus, these findings suggest that *DLX5* and *DLX6* are maternally imprinted genes. Interestingly, *DLX5* was previously shown to be expressed only from the maternal allele in brain and peripheral blood cells.^{21 22} We studied allelic expression of *DSS1*, *DLX5* and *DLX6* in cultured osteoblasts obtained from the right femur of an unaffected individual. We found that all three genes were expressed only from the paternal allele, confirming that all the three genes are maternally imprinted in osteoblasts.

Since the *DSS1* RNA and protein levels of our proband were not different from those of the control, this suggested that DNA

in the deleted region did not regulate the expression of *DSS1*. On the contrary, the RNA and protein of *DLX5* and *DLX6* of our proband were absent, suggesting that enhancers of these two genes were in the deleted region. This confirms the role of *DYNC111* exonic enhancers in normal limb formation in humans.

There were seven previously reported patients in five unrelated pedigrees with SHFM1 who had chromosomal deletions with known parental origins.^{12 23–26} Six inherited the deletions from their fathers. There was only one patient with a split left hand who inherited the deletion from his non-penetrant mother. Further studies are required to elucidate this unexpected finding.

In summary, we reported the smallest deletion associated with SHFM1, confirmed the role of *DYNC111* exonic enhancers in development of human limb buds and found for the first time that *DSS1*, *DLX5* and *DLX6* were maternally imprinted genes in osteoblasts. SHFM1 in our family is caused by a heterozygous paternal deletion of the enhancers of the osteoblast-specific maternally imprinted *DLX6* and *DLX5* genes, leading to the absence of their proteins.

Acknowledgements We thank the family members for participating in this study, Dr Noppachart Limpaphayom and Dr Kawee Pataradool for collecting bone samples.

Contributors SR performed molecular genetics and functional studies and drafted the manuscript. ST performed mutation analysis. CS performed quantitative real-time PCR analysis. PK coordinated sample collection and collected the clinical data. KS and VS designed, supervised the study and participated in the draft of the manuscript. All authors read and approved the final manuscript.

Funding This study was supported by the Royal Golden Jubilee Ph.D. Program to SR (grant no. PHD/0071/2554), the Ratchadapiseksomphot Endowment Fund of Chulalongkorn University (RES560530177-HR) and the Thailand Research Fund (RTA5680003).

Competing interests None.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Data from this study that do not pertain to individual patients are freely available, in accordance with the principles of the funding agency, and can be obtained by contacting the authors.

REFERENCES

- Gurrieri F, Everman DB. Clinical, genetic, and molecular aspects of split-hand/foot malformation: an update. *Am J Med Genet Part A* 2013;161A:2860–72.
- Elliott AM, Evans JA, Chudley AE. Split hand foot malformation (SHFM). *Clin Genet* 2005;68:501–5.
- Sowinska-Seidler A, Socha M, Jamsheer A. Split-hand/foot malformation—molecular cause and implications in genetic counseling. *J Appl Genet* 2014;55:105–15.
- Gurnett CA, Dobbs MB, Nordsieck EJ, Keppel C, Goldfarb CA, Morcuende JA, Bowcock AM. Evidence for an additional locus for split hand/foot malformation in chromosome region 8q21.11-q22.3. *Am J Med Genet Part A* 2006;140:1744–8.
- Niedrist D, Lurie IW, Schinzel A. 4q32-q35 and 6q16-q22 are valuable candidate regions for split hand/foot malformation. *Eur J Hum Genet* 2009;17:1086–91.
- van Siffhout AT, van den Akker PC, Dijkhuizen T, Verheij JB, Olderoode-Berends MJ, Kok K, Sikkema-Raddatz B, van Ravenswaaij-Arts CM. Split hand/foot malformation due to chromosome 7q aberrations (SHFM1): additional support for functional haploinsufficiency as the causative mechanism. *Eur J Hum Genet* 2009;17:1432–8.
- Birnbaum RY, Everman DB, Murphy KK, Gurrieri F, Schwartz CE, Ahituv N. Functional characterization of tissue-specific enhancers in the *DLX5/6* locus. *Hum Mol Genet* 2012;21:4930–8.
- Merlo GR, Paleari L, Mantero S, Genova F, Beverdam A, Palmisano GL, Barbieri O, Levi G. Mouse model of split hand/foot malformation type I. *Genesis* 2002;33:97–101.
- Robledo RF, Rajan L, Li X, Lufkin T. The *Dlx5* and *Dlx6* homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev* 2002;16:1089–101.
- Shamseldin HE, Faden MA, Alashram W, Alkuraya FS. Identification of a novel *DLX5* mutation in a family with autosomal recessive split hand and foot malformation. *J Med Genet* 2012;49:16–20.
- Wang X, Xin Q, Li L, Li J, Zhang C, Qiu R, Qian C, Zhao H, Liu Y, Shan S, Dang J, Bian X, Shao C, Gong Y, Liu Q. Exome sequencing reveals a heterozygous *DLX5* mutation in a Chinese family with autosomal-dominant split-hand/foot malformation. *Eur J Hum Genet* 2014;22:1105–10.
- Lango Allen H, Caswell R, Xie W, Xu X, Wragg C, Turnpenny PD, Turner CL, Weedon MN, Ellard S. Next generation sequencing of chromosomal rearrangements in patients with split-hand/split-foot malformation provides evidence for *DYNC111* exonic enhancers of *DLX5/6* expression in humans. *J Med Genet* 2014;51:264–7.
- Gurrieri F, Everman DB. Clinical, genetic, and molecular aspects of split-hand/foot malformation: an update. *Am J Med Genet A* 2013;161A:2860–72.
- Kouwenhoven EN, van Heeringen SJ, Tena JJ, Oti M, Dutilh BE, Alonso ME, de la Calle-Mustienes E, Smeenk L, Rinne T, Parsaulian L, Bolat E, Jurgelenaite R, Huynen MA, Hoischen A, Veltman JA, Brunner HG, Roscioli T, Oates E, Wilson M, Manzanares M, Gomez-Skarmeta JL, Stunnenberg HG, Lohrum M, van Bokhoven H, Zhou H. Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. *PLoS Genet* 2010;6:e1001065.
- Crackower MA, Sinasac DS, Xia J, Motoyama J, Prochazka M, Rommens JM, Scherer SW, Tsui LC. Cloning and characterization of two cytoplasmic dynein intermediate chain genes in mouse and human. *Genomics* 1999;55:257–67.
- Sinasac DS, Moriyama M, Jalil MA, Begum L, Li MX, Iijima M, Horiuchi M, Robinson BH, Kobayashi K, Saheki T, Tsui LC. *Slc25a13*-knockout mice harbor metabolic deficits but fail to display hallmarks of adult-onset type II citrullinemia. *Mol Cell Biol* 2004;24:527–36.
- Birnbaum RY, Clowney EJ, Agamy O, Kim MJ, Zhao J, Yamanaka T, Pappalardo Z, Clarke SL, Wenger AM, Nguyen L, Gurrieri F, Everman DB, Schwartz CE, Birk OS, Bejerano G, Lomvardas S, Ahituv N. Coding exons function as tissue-specific enhancers of nearby genes. *Genome Res* 2012;22:1059–68.
- Suzuki K, Haraguchi R, Ogata T, Barbieri O, Alegria O, Vieux-Rochas M, Nakagata N, Ito M, Mills AA, Kurita T, Levi G, Yamada G. Abnormal urethra formation in mouse models of split-hand/split-foot malformation type 1 and type 4. *Eur J Hum Genet* 2008;16:36–44.
- Crackower MA, Scherer SW, Rommens JM, Hui CC, Poorkaj P, Soder S, Cobben JM, Hudgins L, Evans JP, Tsui LC. Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3-q22.1 and analysis of a candidate gene for its expression during limb development. *Hum Mol Genet* 1996;5:571–9.
- Ma YY, Lin H, Chang FM, Chang TC, Trieu T, Pridgen HI, Zhang Y, Huang J, Patino-Guzman K, Diab N, Cantu A, Slaga TJ, Wei SJ. Identification of the deleted in split hand/split foot 1 protein as a novel biomarker for human cervical cancer. *Carcinogenesis* 2013;34:68–78.
- Okita C, Meguro M, Hoshiya H, Haruta M, Sakamoto YK, Oshimura M. A new imprinted cluster on the human chromosome 7q21-q31, identified by human-mouse monochromosomal hybrids. *Genomics* 2003;81:556–9.
- Horike S, Cai S, Miyano M, Cheng JF, Kohwi-Shigematsu T. Loss of silent-chromatin looping and impaired imprinting of *DLX5* in Rett syndrome. *Nat Genet* 2005;37:31–40.
- Asmus F, Hjermdind LE, Dupont E, Wagenstaller J, Haberlandt E, Munz M, Strom TM, Gasser T. Genomic deletion size at the epsilon-sarcoglycan locus determines the clinical phenotype. *Brain* 2007;130:2736–45.
- Haberlandt E, Löffler J, Hirst-Stadlmann A, Stockl B, Judmaier W, Fischer H, Heinz-Erian P, Müller T, Utermann G, Smith RJ, Janecke AR. Split hand/split foot malformation associated with sensorineural deafness, inner and middle ear malformation, hypodontia, congenital vertical talus, and deletion of eight microsatellite markers in 7q21.1-q21.3. *J Med Genet* 2001;38:405–9.
- Marinoni JC, Stevenson RE, Evans JP, Geshuri D, Phelan MC, Schwartz CE. Split foot and developmental retardation associated with a deletion of three microsatellite markers in 7q21.2-q22.1. *Clin Genet* 1995;47:90–5.
- Wieland I, Muschke P, Jakubiczka S, Volleth M, Freigang B, Wieacker PF. Refinement of the deletion in 7q21.3 associated with split hand/foot malformation type 1 and Mondini dysplasia. *J Med Genet* 2004;41:e54.



Absent expression of the osteoblast-specific maternally imprinted genes, *DLX5* and *DLX6*, causes split hand/split foot malformation type I

Sawitree Rattanasopha, Siraprapa Tongkobpetch, Chalurmporn Srichomthong, et al.

J Med Genet published online October 20, 2014
doi: 10.1136/jmedgenet-2014-102576

Updated information and services can be found at:
<http://jmg.bmj.com/content/early/2014/10/19/jmedgenet-2014-102576.full.html>

These include:

Data Supplement

"Supplementary Data"

<http://jmg.bmj.com/content/suppl/2014/10/21/jmedgenet-2014-102576.DC1.html>

References

This article cites 26 articles, 11 of which can be accessed free at:
<http://jmg.bmj.com/content/early/2014/10/19/jmedgenet-2014-102576.full.html#ref-list-1>

P<P

Published online October 20, 2014 in advance of the print journal.

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Advance online articles have been peer reviewed, accepted for publication, edited and typeset, but have not yet appeared in the paper journal. Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

To request permissions go to:
<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:
<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:
<http://group.bmj.com/subscribe/>

**Topic
Collections**

Articles on similar topics can be found in the following collections

[Genetic screening / counselling](#) (800 articles)
[Immunology \(including allergy\)](#) (547 articles)
[Molecular genetics](#) (1174 articles)

Notes

Advance online articles have been peer reviewed, accepted for publication, edited and typeset, but have not yet appeared in the paper journal. Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

To request permissions go to:

<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:

<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:

<http://group.bmj.com/subscribe/>