Copy-number variants and candidate gene mutations in isolated split hand/foot malformation

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Abstract

Split hand/foot malformation (SHFM) is a congenital limb deficiency with missing or shortened central digits. Some SHFM genes have been identified but the cause of many SHFM cases is unknown. We used single nucleotide polymorphism (SNP) microarray analysis to detect copy-number variants (CNVs) in 25 SHFM cases without other birth defects from New York State (NYS), prioritized CNVs absent from population CNV databases, and validated these CNVs using qPCR. We tested for the validated CNVs in seven cases from Iowa using qPCR, and also sequenced 36 SHFM candidate genes in all subjects. Seven NYS cases had a potentially deleterious variant: two had a p.R225H or p.R225L mutation in *TP63*, one had a 17q25 microdeletion, one had a 10q24 microduplication, and three had a 17p13.3 microduplication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Additionally, one Iowa case had a *de novo* 10q24 microduplication. The 17q25 microdeletion has not been reported previously in SHFM and included two SHFM candidate genes (*SUMO2* and *GRB2*), while the 10q24 and 17p13.3 CNVs had breakpoints within genomic regions that contained putative regulatory elements and a limb development gene. In SHFM pathogenesis, the microdeletion may cause haploinsufficiency of SHFM genes and/or deletion of their regulatory regions, and the microduplications could disrupt regulatory elements that control transcription of limb development genes.

Keywords

congenital abnormalities; congenital limb deformities; copy number variation; ectrodactyly; split hand foot malformation; TP63 gene

INTRODUCTION

Split hand/foot malformation (SHFM) is a distal limb malformation involving missing or shortened central digits, often in association with fusion of the remaining digits and median clefts of the hands and/or feet.[1] SHFM has an estimated prevalence of 1–9 per 100 000 births,[2–4] represents 1–15% of congenital limb deficiencies,[2–4] occurs in non-syndromic and syndromic forms,[5] and displays phenotypic variability even within families.[6] Loss of the central portion of the apical ectodermal ridge (AER), a critical signaling center for distal limb outgrowth and digit development located at the apex of the limb bud,[7] is thought to be responsible for SHFM.[8] In humans, the AER is visible in the developing upper limb by embryonic day (E) 32 after conception (gestational week 7).[9,10] The bones and musculature of the human limb are established by E56 (gestational week 10);[10] therefore, SHFM likely occurs during the seventh to ninth weeks of gestation.

Multiple genetic loci have been associated with SHFM, including mutations in *TP63*,[5] *WNT10B*,[6] *CDH3*,[11] *DLX5*,[12] *FGFR1*,[13] *FGFR2*,[14] and *MAP3K20*.[15] Reported copy-number gains at chromosome 10q24[16] and 17p13.3,[17] microdeletions at chromosome 2q31,[18] chromosome rearrangements at 2q14[19] and 6q21,[20] and linkage to Xq26 [21] in SHFM patients suggest that more SHFM genes, as yet undetermined, are present at these and other loci. Because our knowledge of the genetic causes of SHFM is incomplete, we scanned genome-wide for copy-number variants (CNVs) and performed targeted sequencing of candidate genes to search for genetic variants involved in SHFM.

MATERIALS AND METHODS

Subjects

New York State (NYS) has mandatory reporting of major structural birth defects identified within the first two years of life to the NYS Congenital Malformations Registry (CMR).[22] Each birth defect is coded using the expanded British Paediatric Association (BPA) coding system based on hospital-provided descriptions entered as a text field and reviewed by a clinician as needed. We searched the CMR for isolated SHFM cases, here defined as subjects who had SHFM without additional major structural birth defects. We queried CMR

records using BPA codes corresponding to congenital absence of fingers (755.247), congenital cleft hand (755.250), absence of preaxial fingers (755.2609), absence of postaxial fingers (755.2709), congenital absence of foot or toes (755.3401), congenital absence of toe (755.3409), congenital cleft foot (755.350), and absent digits – not otherwise specified (755.440). We selected cases that had ectrodactyly, cleft hand and/or foot, or absent central fingers/toes/digits/phalanges mentioned in the narrative description of the limb defect in the CMR record and that did not have BPA codes indicating the presence of other major birth defects or chromosomal anomalies. In total, 25 isolated SHFM cases were identified from all live births occurring in NYS from 1998–2005 (n = 2 023 049). We also selected five controls with no known major birth defects from among NYS live births delivered during the same time period to use as technical controls for microarray genotyping.

We used single nucleotide polymorphism (SNP) microarrays to detect CNVs and quantitative real-time polymerase chain reaction (qPCR) assays to validate CNVs in NYS cases and controls, and used another group of SHFM cases and controls from Iowa to test for CNVs that we validated in NYS SHFM cases. Seven isolated SHFM cases and seven controls without major birth defects, and their parents, were available from among live births delivered from 1999–2009 in Iowa. The medical records of the Iowa cases were reviewed by clinical geneticists to confirm the diagnosis of SHFM and the absence of other major birth defects. Iowa subjects were examined for validated CNVs using qPCR assays. All NYS and Iowa cases and controls were included in targeted sequencing assays of SHFM candidate genes.

The NYS Department of Health Institutional Review Board, the University of Iowa Institutional Review Board, and the National Institutes of Health – Office of Human Subjects Research Protections approved this study.

DNA specimens

For each NYS case and control, DNA was obtained from residual blood spots archived by the NYS Newborn Screening Program. The DNA was extracted from two 3-mm dried blood spot punches using a laboratory-developed method.[23] For Iowa case- and control-parent trios, DNA that had been extracted from buccal swabs due to the families' participation in the National Birth Defects Prevention Study[24] was used.

Genotyping

DNA specimens from NYS cases and controls were used for microarray genotyping. Genotyping was performed at the Johns Hopkins University SNP Center using the HumanOmni2.5–4 array and the Infinium HD assay protocol (Illumina, San Diego, CA, USA). One control specimen and one case specimen were run in duplicate and served as quality control specimens. Data were analyzed using Illumina GenomeStudio version 2011.1. The genotype no-call threshold was set at < 0.15. Genotypes were called using genotype clusters defined based on a) the standard cluster file provided by Illumina and b) the data generated in this project. For each of the two sets of genotype calls, genotypes were manually reviewed, re-clustered, edited, and excluded (where appropriate) based on parameters and quality control metrics described in Illumina's Infinium Genotyping Data

Analysis Technical Note (http://res.illumina.com/documents/products/technotes/technote_infinium_genotyping_data_analysis.pdf).

CNV calling and annotation

CNVs were imputed from both sets of SNP genotype data (standard cluster file and custom cluster file) using Illumina's cnvPartition algorithm (version 3.1.6) and the PennCNV algorithm.[25] Each CNV call required a threshold of three SNP probes. For both cnvPartition and PennCNV, the data were GC wave-adjusted to reduce false positive calls. Default confidence values were used: 35 for cnvPartition and 10 for PennCNV. CNV call files were compiled and annotated, and the percent overlap with each of the following control databases was assessed: HapMap common CNVs,[26] Children's Hospital of Philadelphia (CHOP) database of CNVs in healthy individuals,[27] and the Database of Genomic Variants (DGV).[28] The following were also noted: the percent agreement between cnvPartition calls and PennCNV calls, the number of cases and controls with the same/overlapping CNVs, and the transcripts and genes encompassed by each CNV. Transcripts and genes were identified using GENCODE Genes track (version 12, HAVANA and Ensembl Datasets).[29] CNV calls were reviewed for overlap with genes in the Online Mendelian Inheritance in Man (OMIM) database,[30] pathogenic CNVs defined by the Internal Standards for Cytogenomic Arrays database[31] (accessed via the University of California – Santa Cruz (UCSC) Genome Browser[32]), CNVs previously reported in SHFM cases, genes associated with SHFM, and variants in the DECIPHER database[33] associated with phenotype descriptions that included SHFM.

CNV validation

CNVs were selected for validation if they a) had been detected in SHFM, either in previous reports or the DECIPHER database, b) overlapped genes mutated in SHFM, or c) overlapped the coding region of at least one gene in one or more NYS SHFM cases, were not present in NYS controls, and contained at least one gene that was not overlapped by CNVs reported in population databases of CNVs (HapMap, CHOP database, and DGV). The procedure for validation by qPCR is detailed in the Supplementary Methods.

Targeted sequencing of SHFM candidate genes

Forty-nine candidate genes were selected for sequencing because mutations in these genes have been detected in SFHM cases or other patients with congenitally missing digits, the genes are located in or near CNVs or other chromosomal rearrangements detected in cases in this study or in previous reports, or disruption of the genes results in a reduced number of digits in animal models. Only the coding regions and exon-intron boundaries of the genes were sequenced. Targeted sequencing of DNA specimens from NYS and Iowa cases and controls was performed using a custom Ion AmpliSeq panel (ThermoFisher Scientific, Waltham, MA, USA), and sequence variants were annotated using the ANNOVAR[34] program. Details of sequencing and variant annotation are provided in Supplementary Methods.

Selection of potentially pathogenic sequence variants

Thirteen of 49 genes targeted for sequencing were in CNVs that failed to validate by qPCR in our study subjects, and we eliminated these genes from further analysis. Our analysis used the data from the remaining 36 genes (Supplementary Table S1). Annotated variants were filtered to select only those that met all of the following criteria: a quality score 20, a flow evaluator alternate allele observation (FAO) count 20, an allele frequency in any reference population (obtained from ANNOVAR's popfreq_all_20150413 database which contains allele frequencies compiled from several population databases) < 0.01, absent from control samples, located in exons or canonical splice sites, and variants were non-synonymous, nonsense, frameshift, or in-frame insertions/deletions. An additional, non-synonymous *TP63* variant (p.P417T) that had a minor allele frequency of 0.011 in the Complete Genomics 46 (CG46) database (used by ANNOVAR for annotation) was also selected because *TP63* is a known SHFM gene. We considered that *TP63* p.P417T has a minor allele frequency of 0.0029 in the ExAC database,[35] and the CG46 database's small sample size could be responsible for its higher *TP63* p.P417T minor allele frequency.

Validation of selected sequence variants

Sanger sequencing was used to validate the selected sequence variants. The sequencing procedure is described in the Supplementary Methods and the conditions used for PCR are provided in Supplementary Table S2.

Statistical analysis

We used NYS birth certificates to obtain data on maternal age at delivery, race/ethnicity, education at delivery, parity, and smoking during pregnancy, as well as infant sex for NYS SHFM cases and all NYS live births delivered from 1998–2005. We compared the data between these groups using the chi-squared test, and considered $P < 0.05$ to represent statistical significance.

Bioinformatics analysis

We accessed transcriptome profiling (RNA-seq) data and chromatin immunoprecipitation (ChIP-seq) data on the acetylation of lysine 27 of the H3 histone protein (H3K27ac) for mouse and human limb buds through the National Center for Biotechnology Information – Gene Expression Omnibus (GEO) data repository. The data were generated by Cotney et al.[36] and DeMare et al.,[37] and had GEO accession numbers GSE42237 and GSE42413. The data were viewed using the UCSC Genome Browser.

RESULTS

The estimated prevalence of isolated SHFM cases without other major birth defects in NYS from 1998–2005 was 1.24 per 100 000 (25/2 023 049) live births. Maternal age at delivery, race/ethnicity, education at delivery, parity, smoking during pregnancy, and infant sex did not differ significantly different between NYS cases and all NYS live births during this time period (Supplementary Table S3).

In NYS cases, we observed 30 CNVs that had been reported previously in SHFM or that overlapped the coding region of at least one gene and were absent from NYS controls and from population databases of CNVs. Heterozygous CNVs at three regions (Table 1) were validated by qPCR (targeted genomic loci used for validation are listed in Supplementary Table S4). It is possible that the other 27 CNVs did not validate because of their small size. The median (inter-quartile range) size for the three validated compared to the 27 non-validated CNVs was 175.5 (168.7 – 306.6) kb vs. 29.9 (14.5 – 55.0) kb, respectively ($P =$ 0.011; Wilcoxon rank sum test). Most of the 27 CNVs were probably too small to be reliably imputed from the genotype data, especially because there was noise in clustering genotypes on a small number of samples (25 cases and 5 controls). Despite their small size, we attempted to validate the 27 CNVs because we did not want to overlook a CNV that intersected a potentially novel SHFM gene.

Five NYS cases had a validated CNV. One case had a 10q24 duplication (Supplementary Figure S1), another three cases each had a 17p13.3 duplication (Supplementary Figure S2), and an additional case had a 17q25 deletion (Supplementary Figure S3) not described previously in SHFM; the 17q25 CNV region contained 11 genes (Supplementary Table S5). With the use of qPCR, we tested for CNVs at the 10q24, 17p13.3, and 17q25 loci in Iowa cases and controls. One of the Iowa cases and none of the Iowa controls had a 10q24 duplication that overlapped the genomic location of the 10q24 CNV in the NYS case (Supplementary Table S4). Evaluation by qPCR detected no CNVs at 10q24 in the parents of the Iowa case, indicating that the duplication was *de novo*. Analysis of three polymorphic microsatellite markers in this family did not demonstrate inconsistency with Mendelian inheritance. None of the Iowa cases and controls had CNVs in the 17p13.3 or 17q25 region.

To determine whether our SHFM cases had other point mutations that might cause SHFM, we performed targeted next-generation sequencing of 36 SHFM candidate genes (listed in Supplementary Table S1) in NYS and Iowa cases and controls. In cases, we prioritized 38 sequence variants that met our criteria for potential pathogenicity. Fourteen variants, all non-synonymous and heterozygous, were validated by Sanger sequencing (Table 2; Supplementary Figure S4). Two NYS cases had private variants at amino acid R225 of *TP63*; R225 is in the DNA-binding domain of *TP63* and is highly conserved based on multiple alignment of the TP63 protein from 10 vertebrates (Supplementary Figure 5a). Eight mutation predictor tools were used to evaluate the 14 validated variants for potential functional impact, and all predicted that the two R225 variants would be damaging (Table 2; Supplementary Table S6). Another validated variant in *TP63*, p.P417T, also modifies a conserved residue (Supplementary Figure 5b) and is located in a proline-rich region between the tetramerization and sterile alpha motif domains (based on protein domains reported for the TP63 protein with GenBank accession number NP_001108452). Seven mutation predictor tools predicted a deleterious effect of this variant but one tool predicted low functional impact (Supplementary Table S6). For the other 11 variants, the number of tools predicting a deleterious effect ranged from one to five (Table 2; Supplementary Table S6). Because mutation predictor tools lacked consensus on the potential pathogenicity of the latter 12 variants, we did not focus on these variants as potential causes of SHFM.

To delineate the critical regions of the 17q25, 10q24, and 17p13.3 CNVs in SHFM, we compared the genomic coordinates of the validated CNVs from this study with those of previous SHFM reports and DECIPHER database cases that had a limb phenotype description consistent with SHFM. The 17q25 copy-number loss in patient 16 partially overlapped a microdeletion in a patient from the DECIPHER database (Figure 1). The overlap extended from the second intron of *ARMC7* to the seventh intron of *NUP85*. Chromosome 10q24 CNVs were copy-number gains that, except for two CNVs, had at least one breakpoint within the region that extended from *LBX1* to *FGF8* (Figure 2; genomic coordinates and references listed in Supplementary Table S7). Chromosome 17p13.3 CNVs were copy-number gains (with one exception) that either overlapped the region extending from *ABR* to the intergenic region immediately downstream of *TUSC5*, or had a breakpoint within this region (Figure 3; genomic coordinates and references provided in Supplementary Table S8). Genes in all three CNV regions are expressed in developing human limb at E44 (Supplementary Figures S6–S8).

The evolutionarily conserved gene order of the *Lbx1-Fgf8* region led to the recognition that this region contains a group of predicted enhancers, arranged in a precise spatial pattern, that coordinately control *Fgf8* expression during embryonic development.[38–40] Chromosomal rearrangements of this region engineered in mouse embryos suggested that copy-number gains at 10q24 might cause SHFM by disrupting the spatial arrangement of the enhancers leading to dysregulation of gene expression.[40] In human limb buds, the subgroup of predicted enhancers shown to drive reproducible patterns of reporter gene expression in transgenic mouse embryos (Supplementary Table S9) coincided with peaks of histone H3K27ac modifications, often found near active enhancers[41] (Supplementary Figure S9). This provides support for the involvement of predicted enhancers at *LBX1-FGF8* in regulating gene expression during limb development. Because chromosome 17p13.3 copy-number gains are also associated with SHFM, we hypothesized that these CNVs could be disrupting regulatory elements in the 17p13.3 region. Gene order in the *ABR-TUSC5* region is conserved among vertebrates (Supplementary Figure S10), implying that if regulatory elements are located in this region, their spatial orientation could be relevant to their regulation of gene expression. In human and mouse limb buds, there were peaks of histone H3K27ac modifications in conserved, non-coding regions at *ABR-TUSC5* (Figure 4, Supplementary Figure S11). Also, in mouse limb buds, H3K27ac peaks aligned with DNase I hypersensitivity sites that mark open chromatin (Supplementary Figure S11). These observations suggested that some of the conserved, non-coding elements in the *ABR-TUSC5* region could be putative regulatory elements (Supplementary Table S10) that control gene expression in the developing limb.

DISCUSSION

In our population-based study, the prevalence of isolated SHFM without other birth defects was 1.24 per 100 000 live births in NYS. Our study included only isolated cases whereas other studies of SHFM prevalence also considered cases that had other birth defects (including cases with known syndromes); therefore, our prevalence estimate is in the lower range of 1–9 per 100 000 births previously reported for non-syndromic and syndromic SHFM combined.[2–4] A study in Manitoba, Canada[42] is used here as an example for

comparing SHFM prevalence between our study and others. The Manitoba study reported a SHFM prevalence of 5 per 100 000 births between 1957 and 2003.[42] This prevalence estimate was higher than ours but the SHFM cases in the Manitoba study included fetal deaths and live births with known syndromes or multiple birth defects whereas our study was restricted to live births without known syndromes or other birth defects.

Our genome-wide search for CNVs in 25 cases, targeted CNV screening in seven cases, and candidate gene sequencing in all 32 cases, resulted in the identification of a novel 17q25 microdeletion in one case (3%), 10q24 microduplications in two cases (6%), 17p13.3 microduplications in three cases (9%), and potentially damaging mutations in the *TP63* DNA-binding domain in two cases (6%). Our single case with a *de novo* 10q24 CNV adds to three other reports of *de novo* 10q24 microduplications in SHFM.[16,43,44] In previous reports that had samples sizes ranging from 22 to 72 non-syndromic SHFM cases or case families, the percentage of cases with 10q24 copy-number gains was 6–23%,[43,45,46] with 17p13.3 copy-number gains was 18–51%,[45–47] and with *TP63* mutations was 0–11%.[5,45,48] These reports did not use population-based case groups, and some reports included case families selected because linkage to 10q24[43] or 17p13.3[45] had been detected previously. Therefore, variation in case ascertainment methods might account for the differences in percentages among previous reports, and between our study and previous reports.

Consistent with previous reports,[5] the *TP63* mutations detected in SHFM cases in this study were in the DNA-binding domain. Mutations in this domain impair the ability of TP63 protein to bind DNA and regulate transcription.[49] The role of *TP63* in SHFM may be related to the requirement for *TP63* in the stratification of epithelial cells.[50] The AER is a band of stratified epithelium at the distal edge of the limb bud,[7] and in mouse embryos homozygous for *Tp63* with a disrupted DNA-binding domain, the AER appeared poorly stratified and failed to form a distinct epithelial multilayer, and the limbs were truncated or absent at birth.[51–53]

The microdeletion at 17q25 affected 11 genes from *ARMC7* to *GRB2*. For two of the genes, *SUMO2* and *GRB2*, there is evidence for a role in limb development. TP63 protein is able to be sumoylated by SUMO2, a protein that effects post-translational modification, and sumoylation modulates TP63 protein stability.[54] Further, a *TP63* p.Q634X mutation detected in SHFM[5] inhibits TP63 protein sumoylation and negatively affects the ability of *TP63* to regulate transcription.[55] The binding of *GRB2* to *FGFR2*, a SHFM gene[14] that encodes a receptor tyrosine kinase involved in fibroblast growth factor signaling, regulates downstream signaling through *FGFR2*.[56] Possible mechanisms for how the microdeletion leads to SHFM include deleting regulatory regions of SHFM genes, causing haploinsufficiency of SHFM genes, and unmasking damaging, recessive variants in either SHFM genes or SHFM regulatory regions on the other chromosome.

At chromosome 10q24, CNVs overlapped the *LBX1-FGF8* region proposed to contain a series of enhancer elements that control *FGF8* expression.[38–40] *FGF8*, a ligand involved in fibroblast growth factor signaling, is expressed in the AER throughout its existence[57] and is needed to maintain the AER;[58] loss of function of *Fgf8* in the AER of mouse embryos results in missing or shortened digits.[59] The consequences of rearranging the predicted

enhancers relative to their presumed target, *Fgf8*, have been explored by generating tandem duplications of the *Lbx1-Fgf8* region in mouse embryos.[40] The duplications placed the *Lbx1* promoter at the genomic position usually occupied by *Fgf8* resulting in ectopic expression of *Lbx1* in structures where *Fgf8* was normally expressed, including the AER.[40] In the *Dactylaplasia* mouse model of SHFM, caused by the insertion of retrotransposons in the *Lbx1-Fgf8* region, there is ectopic expression of the retrotransposon elements within the AER, cell death of the AER, and limb defects similar to SHFM.[60,61] The insertions are approximately 7 kb in length,[60] and are expected to change the spatial arrangement of regulatory elements in the *Lbx1-Fgf8* region relative to target promoters. Thus, findings from the *Dactylaplasia* mouse model support the concept that modifying the spatial organization of regulatory elements in the *LBX1-FGF8* region could be part of the causal mechanism of SHFM due to CNVs at 10q24.[40]

Copy-number gains at 17p13.3 overlapped the *ABR-TUSC5* region. Together, the CNVs, evolutionarily conserved gene order, and preliminary evidence for conserved regulatory elements at 17p13.3 prompted us to hypothesize that elements regulating the expression of a limb development gene were located in this region. As proposed for the *LBX1-FGF8* region, tampering with the spatial arrangement of the regulatory elements could cause dysregulation of gene expression. The target gene(s) of these putative regulatory elements is unknown, but a candidate is the transcription factor, *BHLHA9*, based on its location within the *ABR-TUSC5* region and on reports linking it to limb development. Homozygous mutations in the *BHLHA9* DNA-binding domain cause mesoaxial synostotic syndactyly with phalangeal reduction, Malik-Percin type (OMIM 609432), a disorder with a clinical phenotype that includes shortened phalanges, clinodactyly, and fusion of toes.[62] A homozygous mutation in the DNA-binding domain of *BHLHA9* was also detected in a patient whose clinical features of polydactyly, syndactyly, camptodactyly, and dysplastic nails, resulted in a diagnosis of complex camptosynpolydactyly (OMIM 607539).[63] Moreover, in zebrafish embryos, *Bhlha9* is expressed in the developing fins, and *Bhlha9* knockdown led to shortening of the pectoral fins.[45] The finding that *Bhlha9*-null mice display cutaneous syndactyly because of reduced apoptosis between the digits[64] implicates *BHLHA9* in at least one aspect of limb development, interdigital apoptosis, but greater definition of the role of *BHLHA9* in limb development is needed.

Additional support for the hypothesis that 17p13.3 CNVs lead to SHFM by disturbing the organization of regulatory elements within this region is provided by data showing that 17p13.3 CNVs in SHFM were relatively small in size (mean of 263 kb), overlapped *BHLHA9*, and had breakpoints in or near the *ABR-TUSC5* region.[65] By contrast, in individuals who did not have SHFM but were mostly affected with intellectual disability, 17p13.3 duplications were larger (mean size of 1.1 Mb), only sometimes overlapped *BHLHA9*, and did not interrupt the *ABR-TUSC5* region because the breakpoints of these duplications usually fell outside of this region.[65] The investigators suggested that *BHLHA9* duplication might be necessary for SHFM pathogenesis due to 17p13.3 CNVs but disturbance of regulatory elements near to *BHLHA9* was probably also part of the pathogenic mechanism.

Our study had several strengths. The detection of chromosome microduplications and microdeletions in several SHFM cases highlights the importance of including chromosomal microarray testing as part of the diagnostic assessment of SHFM patients. Also, the 10q24 and 17p13.3 CNVs in our cases were similar to those in previous reports of SHFM,[16,17,43–45,66] adding to the evidence that these CNVs cause SHFM. One Iowa case had a *de novo* 10q24 CNV, confirming previous reports that *de novo* genetic variants are contributors to some cases of SHFM[16,43,44,65] and emphasizing the need to test parental DNA to determine whether potentially causative variants for SHFM are *de novo*, co-segregate with the phenotype, or show incomplete penetrance. Reports of partially penetrant 10q24 and 17p13.3 CNVs in SHFM[67,68] suggest that other modifier variants or genes also contribute to determining whether SHFM occurs. The finding that manifestation of the SHFM phenotype in the *Dactylaplasia* mouse model relies not only on insertions in the *Lbx1-Fgf8* genomic region but also on being homozygous for a recessive allele at a locus on another chromosome[69] further supports the involvement of multiple interacting loci in producing the SHFM phenotype.

This study also had a number of limitations. No medical record data were available to perform a clinical evaluation of cases, and instead, we relied on hospital reporting of birth defects to our registry to assign BPA codes that were used to identify SHFM cases. It is possible that heterogeneity in documenting birth defects among health care institutions and in coding practices among coders affected whether a patient was identified as a SHFM case. In addition, the term "ectrodactyly", used for searching the registry's narrative case description to identify SHFM, does not describe central ray deficiencies exclusively and may not represent solely SHFM. However, only two of the 25 NYS cases had "ectrodactyly" as the only narrative description of SHFM. Another limitation was that the lack of clinical data on our cases made it difficult to determine whether any had SHFM with long bone deficiency (SHFLD); 17p13.3 microduplications overlapping *BHLHA9* have been detected in many previous reports of SHFLD.[45–47] The narrative case description for one of our NYS cases indicated a longitudinal deficiency of the tibula (suggestive of SHFLD) but we did not detect CNVs on chromosome 10 or 17 or mutations in *TP63* in this case. Finally, because DNA specimens were not available for the parents of SHFM cases from NYS, we could not determine whether genetic variants arose *de novo* in those cases.

To conclude, we provided a population-based estimate for the prevalence of isolated SHFM without other major birth defects and detected potentially damaging *TP63* mutations and CNVs in eight of 32 isolated SHFM cases. The 17q25 microdeletion has not been reported previously in SHFM, and two candidate SHFM genes (*SUMO2* and *GRB2*) within the deleted region are worth following up in other populations. The 10q24 and 17p13.3 CNVs were located in genomic regions that share certain characteristics: evolutionarily conserved gene order, putative regulatory elements, and a limb development gene locus. Therefore, the concept that CNVs shuffle the arrangement of regulatory elements leading to dysregulation of gene expression and SHFM, previously proposed for the *LBX1-FGF8* region,[40] might also apply to the *ABR-TUSC5* region. Our findings and those of others on microdeletions[70] and microduplications[40] in SHFM suggest that CNVs can cause SHFM by deleting or disrupting regulatory elements that control gene transcription in the limb bud. Further

investigation is needed to understand how dysregulated gene expression during digit development leads to SHFM pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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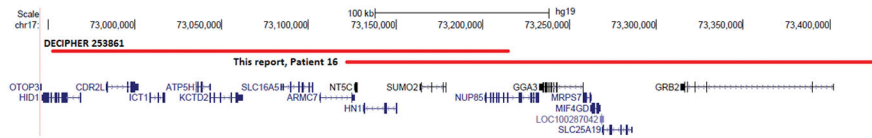
Figure 1.
Region of copy-number loss at chromosome 17q25 in patients with split hand/foot malformation (GRCh37/hg19 assembly). The DECIPHER case was reported with a microdeletion at chr17:72952528-73214654 and the following phenotypes: aplasia of the fingers, cutaneous finger syndactyly, asymmetry of the mandible, and hip dislocation.

Figure 2.
Region of copy-number variation at chromosome 10q24 among patients with split hand/foot malformation (GRCh37/hg19 assembly). All variants were copy-number gains. Breakpoints and references to patient reports are listed in Supplementary Table S7.
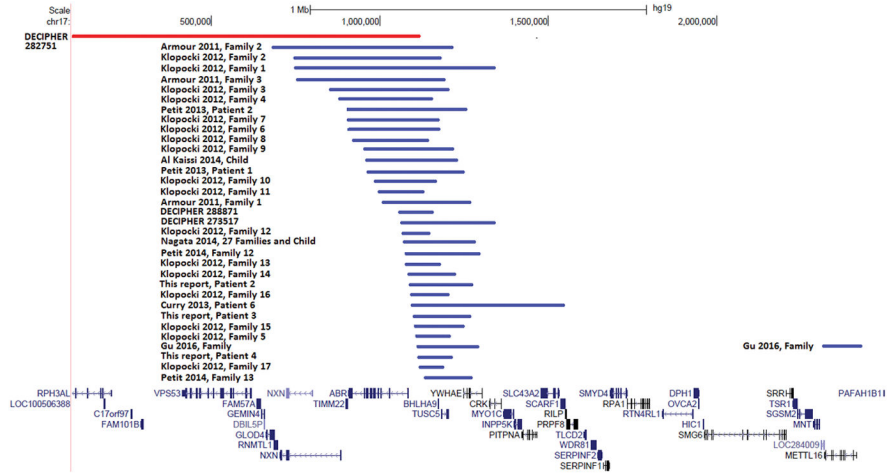
Figure 3.
The overlap of copy-number variation at chromosome 17p13.3 in patients with split hand/ foot malformation (GRCh37/hg19 assembly). The DECIPHER 282751 case had a copy-number loss; all other cases had a copy-number gain. Copy-number variant breakpoints and references to patient reports are listed in Supplementary Table S8.
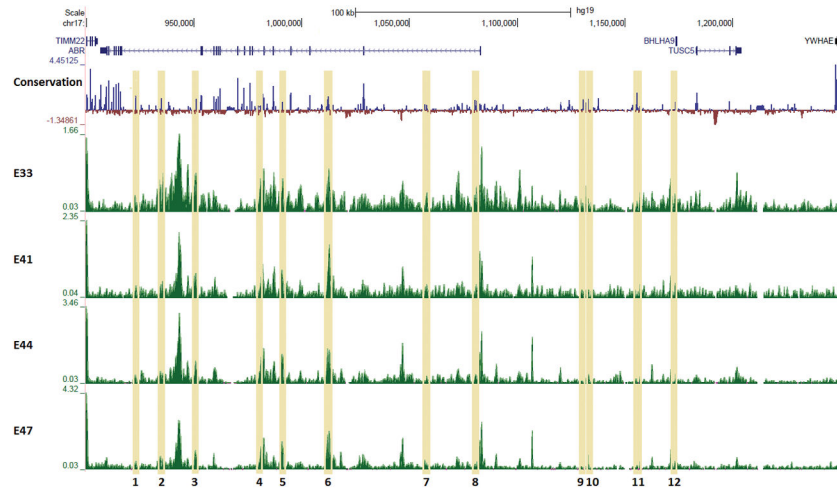
Figure 4.
Identification of putative regulatory elements in the chromosome 17p13.3 region at
chr17:900000-1250000 (GRCh37/hg19 assembly). Histone H3K27ac chromatin
immunoprecipitation data are shown for human limb buds at embryonic day (E) 33, E41,
E44, and E47. Shaded areas highlight peaks of histone H3K27ac modification that align
with peaks of evolutionary conservation (based on multiple alignment of the genomes of 100
vertebrates using the PhyloP method) in non-coding regions. The number below each shaded
area represents the element number in Supplemental Table 9 that lists the genomic
coordinates of the H3K27ac peaks. The histone H3K27ac chromatin immunoprecipitation
data were generated by Cotney et al.[36]

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Table 1

Copy number variants detected and validated in New York State split hand/foot malformation cases

Cytogenetic band	Genomic coordinates[1]	Size (kilobases)	Copy number variation	Patient number
10q24	chr10:102969972-103452645	482.7	Duplication	1
17p13.3	chr17:1087227-1267395	180.2	Duplication	2
17p13.3	chr17:1098724-1263590	164.9	Duplication	3
17p13.3	chr17:1114910-1211121	96.2	Duplication	4
17q25	chr17:73121409-73428037	306.6	Deletion	16

[1]Coordinates are based on the GRCh37/hg19 human genome assembly.

Table 2

Validated sequence variants in patients with split hand/foot malformation

Gene	GenBank mRNA accession number	Protein change	Maximum minor allele frequency[1]	dbSNP accession number	Number out of eight mutation prediction tools that predicted a functional effect	Patient number
TP63	NM_001114980	p.R225H	NA	NA	8	5[2]
TP63	NM_001114980	p.R225L	NA	NA	8	6[2]
TP63	NM_001114980	p.P417T	0.011	rs148076109	7	7[2]
EVX2	NM_001080458	p.A472T	0.0006	rs368732107	1	2[3]
HOXD12	NM_021193	p.N237T	0.0023	rs199589140	5	8
HOXD11	NM_021192	p.G245D	0.0002	rs376305712	3	6[2]
HOXD10	NM_002148	p.L57P	0.0002	rs201449517	3	9
HOXD3	NM_006898	p.G42S	0.0053	rs138422926	1	10
HOXD1	NM_024501	p.G218R	0.0039	rs150112597	3	11
FGFR1	NM_001174066	p.P283S	0.0001	rs377648976	2	12
ROR2	NM_004560	p.D895G	0.0008	rs149826387	1	13
POLL	NM_001174084	p.E498K	0.0002	rs377327286	5	5[2]
CDH3	NM_001793	p.R175W	0.0001	NA	5	27
CDH3	NM_001793	p.M269L	0.0072	rs36038900	2	7[2]

Abbreviation: NA, Not available.

[1] Minor allele frequency obtained from ANNOVAR's [34] popfreq_all_20150413 database which contains allele frequencies compiled from several population databases.

[2] Patient had two sequence variants.

[3] Patient also had a validated copy-number variant at chromosome 17p13.3.