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Exome Sequencing to Identify Novel Susceptibility Genes for Nonsyndromic Split-Hand/Ft Malformation: A Report From the National Birth Defects Prevention Study

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Author Contributions

Tonia Carter performed the analysis, interpreted data, and wrote the initial draft of the manuscript. Denise Kay and Faith Pangilinan assisted with laboratory methods and data interpretation. Lynn Almi and Mary Jenkins assisted with study design and data interpretation. Elizabeth Blue, Pagna Sok, and Janson White assisted with data analysis methods and data interpretation. Other authors contributed to funding acquisition, study design, data collection, and data interpretation. All authors reviewed and edited the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Ethics Statement

The Centers for Disease Control and Prevention Institutional Review Board, along with the Institutional Review Boards for each participating study site, has approved the NBDPS.

Consent

All interviewed study participants provided informed consent.

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Abstract

Background: Split-hand/foot malformation (SHFM) is a rare, genetically heterogeneous, congenital limb defect. Some but not all associated genes are known; therefore, the aim was to identify genes underlying SHFM.

Methods: Buccal cell-derived DNA from 26 children with SHFM and their parents who participated in the National Birth Defects Prevention Study was exome sequenced. Family-based trio analyzes prioritized rare coding variants by inheritance patterns, predicted pathogenicity, and location within putative limb development genes. Copy-number variants in SHFM candidate genomic regions were also examined. Case-control analyzes compared coding variants between case children and 1191 controls (parents of children with non-limb birth defects). Variant validation was by Sanger sequencing or droplet digital polymerase chain reaction.

Results: In family-based analyzes, the prioritized and validated variants (each in a single family) included likely damaging variants that were heterozygous and *de novo* in speckle type BTB/POZ protein (*SPOP*) and ubiquitin-like modifier activating enzyme 2 (*UBA2*), X-linked recessive in fibroblast growth factor 13 (*FGF13*) and RNA binding motif protein 10 (*RBM10*), and compound heterozygous in interleukin enhancer binding factor 3 (*ILF3*). Validation assays did not confirm predicted *de novo* copy-number gains at chromosomes 10q24 and 19p13.11. Case-control analyzes did not identify statistically significant associations.

Conclusion: Exome analysis nominated new susceptibility genes (*FGF13*, *ILF3*, *RBM10*, *SPOP*) and detected a variant in a known candidate gene (*UBA2*). Follow-up investigation is needed to ascertain damaging variants in these genes in additional cases with SHFM and evaluate the impact of the variants on gene expression, protein function, and limb development.

Keywords

birth defects; congenital limb malformation; ectrodactyly; exome sequencing; NBDPS; split foot; split hand

1 | Introduction

Split-hand/foot malformation (SHFM) is a rare congenital limb defect, most commonly involving shortened or missing central digits in the hands and/or feet, sometimes with fusion of the remaining digits or a deep, central cleft in the hand and/or foot (Kantaputra and Carlson 2019). The estimated prevalence is 1–6 per 100,000 births (Bedard et al. 2015; Evans et al. 1994). SHFM can occur as an isolated defect (Akimova et al. 2023), in combination with other birth defects (e.g., congenital heart defects and craniofacial anomalies) (Elliott and Evans 2008; Rasmussen et al. 2016), or as part of a syndrome (Palumbo et al. 2019), and has been reported recurrently in at least 50 genetic syndromes (Umair and Hayat 2020). SHFM can be sporadic or inherited (Petit et al. 2014), and the mode of inheritance can be autosomal dominant, autosomal recessive, or X-linked (Umair and Hayat 2020). Studies of families with SHFM interrogated by genetic testing indicate that penetrance is often incomplete (Rattanasopha et al. 2014; Ugur and Tolun 2008). The phenotypic expression of SHFM also can be extremely variable among members of the same affected family, ranging from syndactyly to SHFM of all four limbs (Dai et al. 2013; Khan et al. 2012; Sowinska-Seidler et al. 2014).

The pathogenic mechanisms of SHFM are not fully known but are thought to be driven by abnormalities in embryonic limb outgrowth (Gurrieri and Everman 2013; Kantaputra and Carlson 2019). Limb bud outgrowth is controlled by interactions between signaling pathways in the apical ectodermal ridge, a highly specialized layer of epithelial cells at the distal tip of the limb bud, and the underlying mesenchyme (Mariani et al. 2017). The signaling interactions regulate cell proliferation and differentiation (Zuniga and Zeller 2020), and the disruption of these interactions or failure to maintain the apical ectodermal ridge is thought to lead to SHFM (Gurrieri and Everman 2013).

SHFM is genetically heterogeneous, with at least 16 loci implicated in case reports and case series (Barnett et al. 2016; Begemann et al. 2015; Faiyaz ul Haque et al. 1993;

Holder-Espinasse et al. 2019; Kjaer et al. 2005; Low and Nwbury-Ecob 2012; Simonis et al. 2013; Truong et al. 2023; Umair and Hayat 2020; Yamoto et al. 2019). Several of these loci have support from animal models or display spatiotemporal expression patterns during embryogenesis that suggest roles in limb development (Table S1).

Only a fraction of all individuals with SHFM have been screened by genetic testing and reported in the literature, making it likely that additional genes involved in SHFM remain to be revealed. Given that limb development is a complex process involving many interacting genes, the 16 implicated loci probably form only a partial list of genetic contributors to SHFM. New SHFM loci continue to be detected with the increasing use of exome/genome sequencing and other types of genetic testing in SHFM (de Boer et al. 2023; Dufour et al. 2022; Odrzywolski et al. 2024; Peluso et al. 2021). Identifying more of the loci involved in SHFM would pave the way for an increased understanding of the molecular mechanisms that result in SHFM. This study aimed to discover genes involved in the pathogenesis of SHFM. Therefore, we used a population-based sample of case-parent trios to perform an exome sequencing analysis to identify susceptibility genes for SHFM.

2 | Materials and Methods

2.1 | Study Participants

We selected study participants from the National Birth Defects Prevention Study (NBDPS), a population-based study to assess risk factors for > 30 major structural birth defects (Yoon et al. 2001). A description of NBDPS methods has been published previously (Reefhuis et al. 2015). Birth defects surveillance systems in 10 US states (Arkansas, California, Georgia, Iowa, Massachusetts, New Jersey, New York, North Carolina, Texas, and Utah) ascertained cases with eligible defects among pregnancies that ended on or after October 1, 1997, or had an estimated delivery date on or before December 31, 2011. To confirm eligibility for the NBDPS, a clinical geneticist at each NBDPS site reviewed the abstracted medical record data of cases. A clinical geneticist performed further review of clinical information to classify cases using standard case definitions across NBDPS sites (Rasmussen et al. 2003). SHFM was defined as complete or partial absence of one or more of the central rays of the hand or foot (fingers/toes and metacarpals/metatarsals 2–4, or monodactyly). Cases with SHFM were classified as having defects that were isolated (presence of one major defect) or multiple (presence of two or more unrelated, major defects in different organ systems). Cases with chromosomal abnormalities or known syndromic or single-gene disorders were not included in NBDPS.

Following the completion of a computer-assisted telephone interview within 6 weeks to 2 years after their estimated dates of delivery, mothers were mailed a kit with cytobrushes to self-collect buccal cell samples from themselves, their child (if living) and the child's father. Mothers were excluded if they had already participated in the NBDPS with a previous pregnancy, could not complete the interview in English or Spanish, were imprisoned, or did not have legal custody of their child at the time of study enrollment. We selected participants who were the unaffected parents of NBDPS cases with non-limb birth defects and had provided buccal cell samples that were used for exome sequencing as controls for a case-control analysis.

2.2 | Buccal Cell Samples

Participants self-collected buccal cell samples using cytobrushes packaged in open paper-backed peel pouches (allowing air drying of the samples) (Gallagher et al. 2011; Jenkins et al. 2019). DNA was extracted from buccal cell samples using several protocols, as previously reported (Jenkins et al. 2019; Rasmussen et al. 2002).

2.3 | Exome Sequencing

Buccal cell-derived DNA samples were sequenced at the National Institutes of Health Intramural Sequencing Center (Rockville, MD; <https://www.nisc.nih.gov/>) in the National Human Genome Research Institute. Exomes were captured using the SeqCap EZ Human Exome + UTR kit v3.0 (Roche NimbleGen, Madison, WI), which targeted 96 megabases of the genome. DNA libraries underwent 126-base pair paired-end read sequencing on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA). We performed image analysis, base calling, and initial mapping of sequence reads to a human reference genome to generate binary alignment map files as described in previous reports (Jenkins et al. 2019; Sok et al. 2023).

2.4 | Exome Sequence Data Processing

After exome sequencing and initial read mapping, the University of Washington Center for Mendelian Genomics reprocessed binary alignment map files, as reported in detail previously (Jenkins et al. 2019; Sok et al. 2023). Briefly, reads were aligned to a human reference genome (hg19hs37d5) using BWA-MEM version 0.7.10 (Li and Durbin 2010), read-pairs not mapping within ± 2 SD of the average library size ($\sim 150 \pm 15$ base pairs for exomes) were removed, and the remaining reads underwent removal of polymerase chain reaction (PCR) duplicates, insertion/deletion (indel) realignment, base quality recalibration, variant detection, and variant quality score recalibration using software tools in the Genome Analysis Toolkit package (McKenna et al. 2010). Variant quality score recalibration involved measuring the quality of all variants using seven annotation profiles: quality by depth, mapping quality, MQRankSum, ReadPosRankSum, Fisher strand, Strand odds ratio, and inbreeding coefficient. We assessed single nucleotide variants (SNVs) and indels independently, as recommended by the Genome Analysis Toolkit, and flagged SNVs and indels below the 99.7 and 99.0 percentiles, respectively, as low quality. We also flagged variants if they had other indicators of being low quality or potential false positives, including a quality score ≤ 50 , long homopolymer run > 4 , quality by depth < 5 , or location within a cluster of SNVs.

2.5 | Family Trio Analysis

The University of Washington Center for Mendelian Genomics performed variant processing, as described previously (Jenkins et al. 2019). The processing pipeline included variant annotation using the ENSEMBL Variant Effect Predictor program (McLaren et al. 2016), variant filtering to retain high-quality variants with an alternate allele frequency ≤ 0.005 in population databases of genetic variants and predicted to have a medium or high impact on protein function, variant classification according to standard Mendelian inheritance models, and copy-number variant (CNV) calling using the Conifer version

0.2.2 program (Krumm et al. 2012). Variants with an autosomal recessive (homozygous or compound heterozygous), *de novo*, or X-linked (*de novo* or recessive) mode of inheritance were selected for further review and prioritized based on predicted pathogenicity. Frameshift variants, inframe indels, canonical splicing variants, and stop-gain variants were predicted to be damaging as well as missense or splice region variants that had a PHRED-scaled Combined Annotation Dependent Depletion (CADD) (Kircher et al. 2014) score ≥ 15 , a SIFT (Ng and Henikoff 2003) score ≤ 0.05 , a PolyPhen-2 (Adzhubei et al. 2010) score ≥ 0.85 , and a Genomic Evolutionary Rate Profiling (GERP) (Davydov et al. 2010) score ≥ 3 (indicating conservation of sequence alignment across multiple species). Genes harboring these variants were prioritized based on evidence of a role in limb development or congenital limb defects in the scientific literature. We excluded variants from further consideration if they were reported as benign or likely benign in the ClinVar database (Landrum et al. 2018). CNVs with *de novo* inheritance and overlap with candidate genes or CNV regions previously reported for SHFM were also prioritized.

2.6 | Droplet Digital PCR

CNVs were validated using droplet digital PCR (ddPCR), performed by ACGT Inc. (Wheeling, IL). SHFM family trio DNA samples and a control human DNA sample were quantitated by the Qubit dsDNA High Sensitivity Assay (Life Technologies). Because the quantitation assay showed the SHFM family trio DNA samples had low initial concentrations (< 1 ng/ μ L), these samples were vacuum concentrated, resulting in an increase in DNA concentration to 1–24 ng/ μ L. TaqMan probes (ThermoFisher Scientific) targeting locations within the CNVs (Table S2) were used for ddPCR. The initial testing of ddPCR was performed using the QuantStudio 3D Reagent Control Kit (Applied Biology) and the TaqMan probes on the control DNA sample diluted to four concentrations: 30, 20, 10, and 5 ng/ μ L. After establishing that the performance of the ddPCR assays was acceptable at 5 ng/ μ L (Table S3), additional ddPCR assays with the TaqMan probes were performed in triplicate using the control sample at 5 ng/ μ L and 1 ng/ μ L. The assays for each probe performed satisfactorily at 1 ng/ μ L (Table S3), allowing ddPCR of the SHFM family trio samples to proceed in triplicate, using DNA at 1 ng/ μ L. The assay reaction mixture contained 7.5 μ L 2X QuantStudio 3D Digital Master Mix v2 (Applied Biology), 0.73 μ L 20 $\times$ TaqMan probe, 0.73 μ L Reference TaqMan assay probe, 4.2 μ L nuclease free water, and 1.6 μ L DNA template (at various concentrations) in a total volume of 14.5 μ L. The ddPCR assays were performed on a QuantStudio 3D Digital PCR System (Applied Biology), and the QuantStudio 3D Control Reagent Kit was used as an internal performance control for each run. The ddPCR assays quantitated the genomic region of interest normalized to RNaseP, an endogenous reference gene known to be present in two copies in the human genome. Results were reported as the target gene:RNaseP ratio. Ratios of 1, 1.5, and 0.5 indicate a normal copy number, a heterozygous duplication, and a heterozygous deletion, respectively, of the target gene.

2.7 | Sanger Sequencing

Sanger sequencing was performed by ACGT Inc. using DNA samples from seven SHFM family trios to validate eight candidate variants and their pattern of inheritance. For each of the eight loci, genomic DNA (> 100 ng/reaction) was amplified by PCR (primers in

Table S4), and the amplified DNA was assayed by bidirectional Sanger sequencing. Two individuals independently reviewed the sequencing results.

2.8 | Filtering of Variants and Samples for Case–Control Analysis

Prior to the analysis comparing cases with SHFM to controls, we filtered the processed exome sequence data to remove variants that were flagged as low quality, were multi-allelic, had an average read depth < 10 , had a call rate < 0.99 , did not meet the joint criteria of having a read depth ≥ 6 and a read depth ≤ 500 (because abnormally high read depth is indicative of alignments arising by chance alone) and a genotype quality ≥ 20 in at least 95% of samples, failed the Hardy–Weinberg equilibrium threshold at $p < 10^{-6}$, or were duplicate variant records. We also removed variants that did not map to uniquely mappable regions of the genome, characterized by a 100-mers mappability score of one (Karimzadeh et al. 2018).

We excluded samples from the analysis if any of the seven evaluated metrics (mean genotype sequencing depth, number of variants called, number of singletons, inbreeding coefficient, heterozygous-to-homozygous ratio, transition-to-transversion ratio, and frequency of missing variants) were beyond ± 6 SD from the mean value for all samples. We also excluded control samples that showed genetic relatedness at the second degree or closer with case children or other controls, determined by kinship estimates generated using KING (Manichaikul et al. 2010). Next, we filtered out of the analysis any variants that became monomorphic after removing these samples.

For the analysis of common variants, we included variants with an alternate allele frequency ≥ 0.05 . For the analysis of rare variants, we included variants that had an alternate allele frequency < 0.05 and were predicted to be damaging (missense, frameshift, stop-gain, stop-lost, inframe indel, and canonical splice variants).

2.9 | Case–Control Analysis

We analyzed common variants separately using logistic regression in PLINK version 1.90 (Chang et al. 2015) to identify individual variants associated with SHFM. For the analysis of rare, predicted damaging variants, we used tests for association with all variants within a gene (gene-based association testing). The subset of variants with an alternate allele in at least one case and one control ($n = 49,036$) was pruned, using PLINK, to retain 32,578 variants in low linkage disequilibrium with each other ($r^2 < 0.2$) for generating principal components (PCs) of unmeasured ancestry structure. All case–control analyses assumed an additive genetic model and included sex and the first three PCs (which, together, explained 68.2% of the variance) as covariates.

The genomic inflation factor was calculated by converting p -values from the case–control association tests to chi-squared statistics with one degree of freedom and dividing the median of the resulting chi-squared statistics by the expected median of a chi-squared distribution with one degree of freedom. Because preliminary results for the genomic inflation factor indicated that population stratification was not sufficiently accounted for in the analysis of rare variants, we applied the PCAmatchR approach (Brown et al. 2021) to select 40 PC-matched controls for each case, based on the first 20 PCs, for rare variant analysis. A plot of the multivariate distance between the matched case children and controls

is shown in Figure S1. As an additional measure to avoid the inflation of test statistics, we restricted rare variant analysis to genes that had at least two variants, a minimum alternate allele count of 10 in the case–control sample, and a minimum alternate allele count of 1 in cases.

We implemented gene-based testing for rare variant analysis using the optimal unified sequencing kernel association test (SKAT-O) in the SKAT version 2.2.5 package in R version 4.1.1 (Lee et al. 2012). SKAT-O aggregates score statistics for individual variants within a region and computes region-based p -values analytically. Because the number of controls was much larger than the number of case children, we ran SKAT-O using the “SKATBinary_Robust” function, which adjusts for unbalanced case–control ratios in gene-based association testing (Zhao et al. 2020). This function uses the saddle point approximation and efficient resampling approaches to calibrate the variance of score statistics for individual variants to calculate region-based p -values more accurately.

For case–control association tests, we used a Benjamini–Hochberg false discovery rate threshold of 0.05 to define statistical significance (Benjamini and Hochberg 1995). For analyses of known SHFM susceptibility genes (Table S1) and variants within those genes, we reasoned that the tests were conducted to confirm associations with known SHFM loci. Therefore, we used an unadjusted p -value of 0.05 as the threshold to assess statistical significance at known SHFM loci.

3 | Results

3.1 | Family Trio Analysis

Among 154 women with eligible pregnancies affected by SHFM, 98 (63.6%) completed the telephone interview (Figure 1). This was similar to the overall proportions of case and control mothers who completed the telephone interview in the NBDPS (67% and 65%, respectively) (Reefhuis et al. 2015). Of the 98 mothers, 53 (54.1%) submitted a buccal cell brush from at least one family member (mother, father, or child), which was a slightly lower proportion than the 65% of case mothers and 59% of control mothers who returned buccal cell brushes in the NBDPS (Reefhuis et al. 2015). Twenty-six SHFM child–parent trios had adequate amounts of DNA (≥ 200 ng for each member of a trio) for sequencing and were included in the study (case families numbered 1–26) (Figure 1). Case phenotypes and demographic characteristics are summarized in Table 1. Three case children also had another congenital anomaly (pyloric stenosis, partial pulmonary venous return, and a perimembranous ventricular septal defect) and were classified as having multiple defects in NBDPS; the remainder were classified as having an isolated defect.

We identified 119 autosomal recessive variants (one homozygous and 118 compound heterozygous), 230 *de novo* variants, and 97 X-linked variants (seven *de novo* and 90 recessive) in 305 genes in the 26 case children. Following an evaluation of the predicted pathogenicity of the variants and a literature review of the genes harboring these variants, we prioritized eight predicted damaging variants in seven genes with evidence of involvement in limb development. We visualized the eight variants in Integrative Genomics Viewer version 2.11.2 (Robinson et al. 2017) to determine if they were present on multiple reads before

performing targeted sequencing to validate the variants. Each gene had a predicted damaging variant in no more than one case child.

Sanger sequencing confirmed six variants in five genes: fibroblast growth factor 13 (*FGF13*), interleukin enhancer binding factor 3 (*ILF3*), RNA binding motif protein 10 (*RBM10*), speckle type BTB/POZ protein (*SPOP*), and ubiquitin like modifier activating enzyme 2 (*UBA2*) (Table 2). Apart from *UBA2*, we did not detect variants in other known SHFM susceptibility genes. The *de novo* variants in *SPOP* and *UBA2* and the variants in *ILF3* were heterozygous; the X-linked variants in *FGF13* and *RBM10* were hemizygous.

3.2 | Copy-Number Variant Analysis

Two heterozygous CNVs overlapping candidate SHFM regions were predicted by Conifer to have occurred *de novo*, each in a single case child (Figure S2). The copy-number gain in case 8 coincided with a chromosome 10q24 region, reported to be duplicated in several cases with SHFM in the literature (Everman et al. 2006; Holder-Espinasse et al. 2019). The copy-number gain in case 9 overlapped the start of the coding region and the first intron of epidermal growth factor receptor pathway substrate 15 like 1 (*EPS15L1*), a candidate SHFM gene located at chromosome 19p13.11 (Umair et al. 2018). ddPCR, with the use of a probe targeting the *BTRC* gene in the chromosome 10q24 region, determined that the *BTRC*:RNaseP ratio (mean $\pm$ SD) in case 8 was 1.187 ± 0.055 , which was lower than 1.5 but higher than that of the parents and the control DNA sample (Table 3). In the context of mosaicism (presence of the variant in a fraction of cells in the individual), a target gene:RNaseP ratio of 1.187 could be consistent with a copy-number gain. It is uncertain if mosaicism would be dependent on cell type in the buccal cell samples, which are composed of epithelial and immune cells (Wong et al. 2022). We did not extract DNA according to cell type or determine cell type fractions in the buccal cell samples, and therefore, could not consider the distribution of cell types in the samples.

An indicator of possible mosaicism in case 8 is an allele balance (defined as the ratio of reads supporting an allele to all reads for the variant, considering only heterozygous variants) that differs between the case and the parents of the case. Therefore, allele balance in the genomic region that included the 10q24 gain and one megabase upstream and downstream of the gain was assessed in members of the case 8 family trio, using exome sequence data (Figure S3). The patterns of allele balance for heterozygous variants in the plots were similar between the case child and the parents, suggesting a similar allele balance among family trio members for variants in the 10q24 region and a lack of mosaicism in this region in the case child. Without evidence of mosaicism, a *BTRC*:RNaseP ratio of 1.187 would not be consistent with a copy-number gain. Thus, the predicted 10q24 gain in case 8 was not considered to be validated by ddPCR. The predicted copy-number gain overlapping part of the *EPS15L1* gene in case 9 had an *EPS15L1*:RNaseP ratio (mean $\pm$ SD) of 0.798 ± 0.005 , which was lower than 1.5 and the ratio in the control DNA sample. Therefore, this predicted variant also was not considered to be validated by ddPCR.

3.3 | Case–Control Analysis

The initial study population for case–control analysis included 26 cases with SHFM and 1206 controls. Twenty-six cases and 1191 controls (Table S5) remained for analysis after excluding six controls that did not meet sample quality control criteria and nine controls that had a second degree or closer relationship with case children or other controls. The case children included 16 males (61.5%) and 10 females (38.5%) while the control group had 596 males (50.0%) and 595 females (50.0%) Sex was not associated with case–control status in bivariate analysis ($p = 0.34$; Pearson chi-squared test). We filtered the initial set of 647,419 variants for case children and controls combined to remove 211,409 (32.7%) variants that did not meet quality control criteria (Figure S4). Of the 436,010 bi-allelic SNVs and indels that remained, 18,372 (4.2%) were rare, predicted damaging variants and 17,884 (4.1%) were common variants.

Case–control analysis proceeded with the numbers of subjects, genes, and variants shown in Table 4. No genes/variants had adjusted p -values below the false discovery rate significance threshold in the analysis of rare or common variants (Figure 2a–c). The top gene/variant was the ELMO domain containing 3 (*ELMOD3*) gene for the analysis of rare, predicted damaging variants (adjusted $p = 0.46$) and a missense variant in the endoplasmic reticulum aminopeptidase 1 (*ERAP1*) gene for the analysis of common variants (adjusted $p = 0.71$) (Table 4). The *ERAP1* missense variant, rs3734016, was three times more frequent in cases vs. controls (19.2% vs. 6.4%) and is predicted by both SIFT and PolyPhen-2 to be tolerated. None of the known SHFM susceptibility genes in Table S1 met the criteria for inclusion in rare variant analysis. Six common variants were detected in four known SHFM susceptibility genes and included in the analysis of common variants (Table S6). The six variants all had unadjusted p -values > 0.05 ; therefore, the association test results for these variants were not considered statistically significant.

4 | Discussion

This study nominates four new SHFM susceptibility genes (*FGF13*, *ILF3*, *RBM10*, and *SPOP*), in which we detected and verified potentially pathogenic variants in single cases with SHFM. Strengthening the argument that the variants in these genes could be novel causes of SHFM were the observations that the *SPOP* variant was absent from the gnomAD database, the variants in the other three genes were rare in the gnomAD database, and none of the variants in the four genes have been reported to date in the ClinVar or PubMed databases (Sayers et al. 2021). We also identified a variant in *UBA2*, supporting previous work linking this gene to SHFM. However, we did not observe associations between common variants in four known SHFM susceptibility genes (*CDH3*, *EPS15L1*, *FGFR1*, and *PRDM1*) and SHFM. *FGF13* is a member of the fibroblast growth factor gene family that has diverse roles in development and metabolism, such as the regulation of cell survival and cell proliferation, and the specification of cell identity (Guillemot and Zimmer 2011). *Fgf13* is expressed in the chick limb bud, and sonic hedgehog, a known regulator of limb morphogenesis, regulates *Fgf13* expression in the limb bud (Munoz-Sanjuan et al. 1999). Moreover, *Fgf13* expression is lost from the chick limb bud in the absence of the apical ectodermal ridge or in wingless or limbless mutant embryos. *FGF13* is located in the

chromosome Xq26 susceptibility locus for SHFM (Faiyaz-Ul-Haque et al. 2005). Our study offers evidence that *FGF13* is a causal gene at this locus.

ILF3, a double-stranded RNA-binding protein, is a component of a heterodimeric complex that participates in the regulation of gene expression (Ting et al. 1998). *ILF3* was shown to modulate ubiquitination mediated by cereblon (Surka et al. 2021), a cullin4 RING E3 ubiquitin ligase substrate-recognition subunit, which when knocked down in zebrafish embryos, leads to defects in pectoral fin development (Ito et al. 2010).

RBM10 is a regulator of splicing (Wang et al. 2013), and *RBM10* loss-of-function variants are associated with TARP syndrome, an X-linked recessive condition with clinical features that include developmental delay, brain abnormalities, facial dysmorphism, and limb anomalies (clinodactyly, syndactyly, and talipes equinovarus) (Kumps et al. 2021). *Rbm10* is expressed in the mouse developing limb, supporting a role for *RBM10* in the limb anomalies observed in TARP syndrome (Johnston et al. 2010).

SPOP, a substrate-recognition subunit of the cullin3 RING-box1 E3 ubiquitin ligase complex, mediates protein ubiquitination (Nagai et al. 1997; Zhang et al. 2023). *Spop* regulates skeletal development, and loss of *Spop* leads to shorter digit bones and reduced bone density in mice (Cai and Liu 2016).

UBA2 is one of the two subunits of a small ubiquitin-like modifier (SUMO)-activating enzyme that binds SUMO1 during the process of SUMOylation, a type of post-translational modification, of target proteins (Desterro et al. 1999). Microdeletions encompassing *UBA2* and frameshift, nonsense, or missense variants in *UBA2* have been reported previously in SHFM (Abe et al. 2018; Elsner et al. 2021; Schnur et al. 2021; Yamoto et al. 2019). In an exome sequencing study that detected potentially damaging *UBA2* variants in 16 individuals from seven unrelated families, only four individuals, each from a different family, had SHFM (Schnur et al. 2021). The study described a spectrum of phenotypes present in the 16 subjects, emphasizing the variable presentation of SHFM in the members of a family who all had the same *UBA2* variant. Incomplete penetrance and variable expressivity of *UBA2* variants are also supported by reports of *UBA2* frameshift variants in other families, with only some family members having SHFM (Parveen et al. 2023; Wang et al. 2020). Animal models also support a role for *UBA2* in limb development. *Uba2* is expressed in the limb buds of mice and the pectoral fins of zebrafish embryos, and *Uba2*-null zebrafish embryos display malformed pectoral fins (Costa et al. 2011; Schnur et al. 2021).

The top hits from the analysis comparing case children with controls were *ELMOD3*, involved in ciliary cargo trafficking (Turn et al. 2022), and a common variant in *ERAP1*, involved in facilitating immune responses (Tiburca et al. 2024). Neither hit reached statistical significance, which is unsurprising considering the small case count and likely under-powered study sample. These two genes have not been implicated previously in SHFM.

In addition, ddPCR did not confirm predicted copy-number gains at two known SHFM susceptibility loci, chromosome 10q24 and *EPS15L1*. The inclusion of only nonsyndromic SHFM in the study perhaps contributed to the observed dearth of predicted damaging

variants in known SHFM susceptibility genes in the case children. Several reports of known SHFM susceptibility genes (e.g., *TP63*, *CDH3*, and *IGF2*) in the literature describe patients who have genetic syndromes, with SHFM being among the clinical phenotypes (Otsuki et al. 2016; Shimomura et al. 2008; Yamoto et al. 2017).

The advantages of using buccal cells as a source of DNA include the collection of the cells by a non-invasive, self-administered technique due to the easy accessibility of the cells in the oral cavity, the relatively low cost of collecting the samples in a large, multi-site epidemiological study, and the ability to obtain genomic DNA of sufficient quantity and quality for sequencing (Le Marchand et al. 2001). Validation studies have also shown that DNA from patient-matched buccal cells and blood cells generates comparable results for the identification of genetic variants (de Vries et al. 1996). The cellular heterogeneity of buccal cells, which contain a mixture of squamous epithelial cells and white blood cells (Wong et al. 2022), can be a limitation in epigenetic studies because epigenetic patterns are known to be cell-type specific (Campbell et al. 2020). Therefore, methods to account for cellular heterogeneity should be employed in epigenetic studies that use buccal cell genetic material (Turinsky et al. 2019). However, in studies focused on genetic sequence, cellular heterogeneity is not expected to influence the results.

The strengths of the study included the use of standard case definitions by clinical geneticists, when reviewing the clinical information of case children, to provide a homogenous case group. NBDPS participants were from geographically and ethnically diverse populations and were ascertained from population-based surveillance systems, as opposed to hospital- or clinic-based ascertainment. Full child–parent trios were included, allowing the identification of *de novo* variants. Moreover, data on pregnancy exposures, lifestyle, and demographic characteristics are available from a telephone interview for use in studies of gene–environment interactions.

The study was limited by its small sample size. Given the genetic heterogeneity of SHFM and the small number of case children, the detection of pathogenic variants in the same gene among multiple case children was unlikely. The case–control analyzes were not well powered to find signals reaching statistical significance, while the unbalanced ratio of case children to controls can inflate Type I error (Zhang et al. 2019). To mitigate the latter effect, we applied the robust version of a gene-based rare variant test that was shown to control Type I error for extremely unbalanced case:control ratios (Zhao et al. 2020). Overall, variants not predicted to be damaging based on *in silico* tools were excluded and, in the family trio analysis, genes without prior evidence of association with limb development were also excluded and could have been missed. Additionally, previous reports of reduced penetrance of variants in SHFM suggest the possibility of SHFM being a consequence of inheriting a pathogenic variant from an unaffected parent in an autosomal dominant manner, but variants with this mode of inheritance were not included in this study.

In conclusion, potentially damaging variants in new SHFM susceptibility genes (*FGF13*, *ILF3*, *RBM10*, and *SPOP*) and a previously reported SHFM gene (*UBA2*) were detected by exome sequencing of family trios with nonsyndromic SHFM. Additional investigation could inform whether other cases with SHFM carry pathogenic variants in the new susceptibility

genes, whether the variants impact gene expression or protein function, and whether animal models of the mutated proteins display phenotypes resembling SHFM. The reduced penetrance and variable expressivity previously described in SHFM suggest that genetic modifiers partly determine whether a SHFM phenotype is expressed; therefore, future studies of SHFM may want to consider the role of modifying factors, including epigenetic, gene–gene, and gene–environment interactions, to elucidate the underlying biological basis for the manifestation of a SHFM phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

The data that support the findings of this study are not publicly available because study participants did not provide consent to share their individual-level data publicly. The study questionnaires and process for accessing the data used in this study is described at <https://www.cdc.gov/birth-defects/php/bd-steps-nbdps-data/index.html>. The code book and analytic code may be made available upon request.

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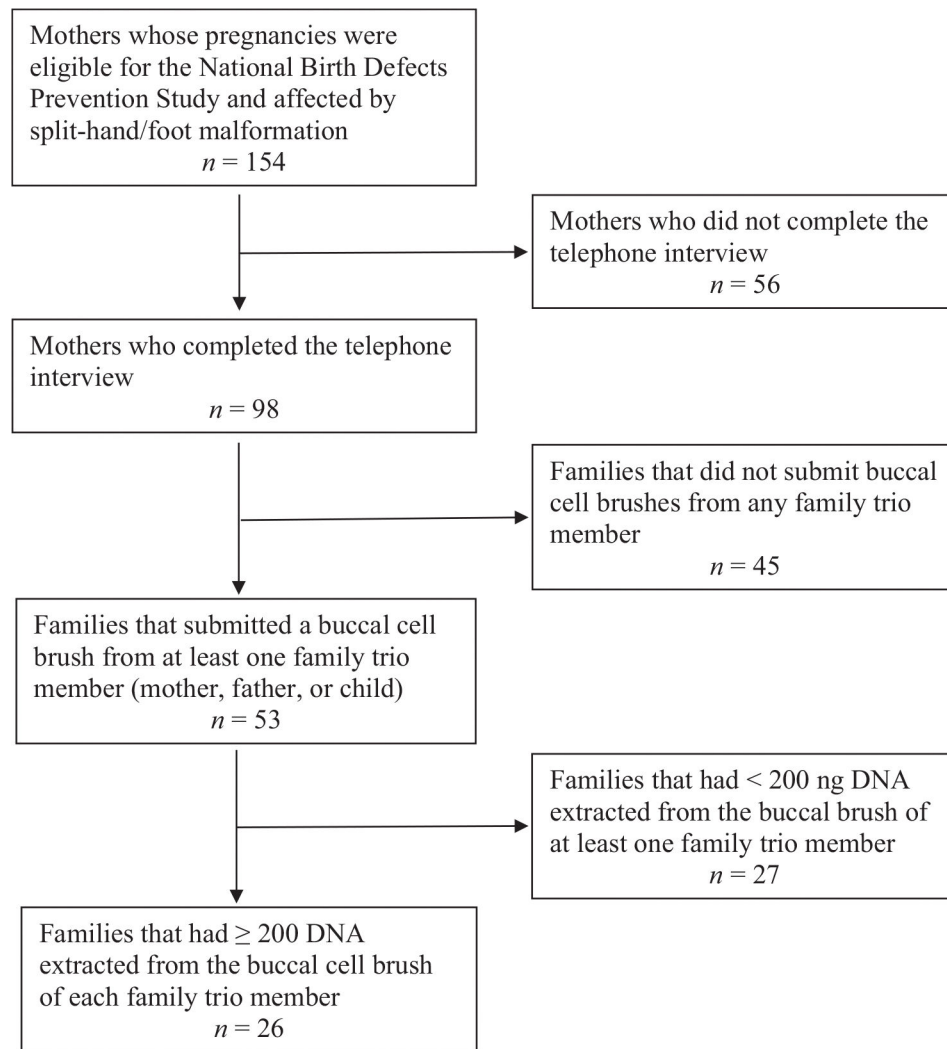


FIGURE 1 |. Flowchart showing the number of split-hand/foot malformation family trios whose DNA samples were exome sequenced in the National Birth Defects Prevention Study, 1997–2011.

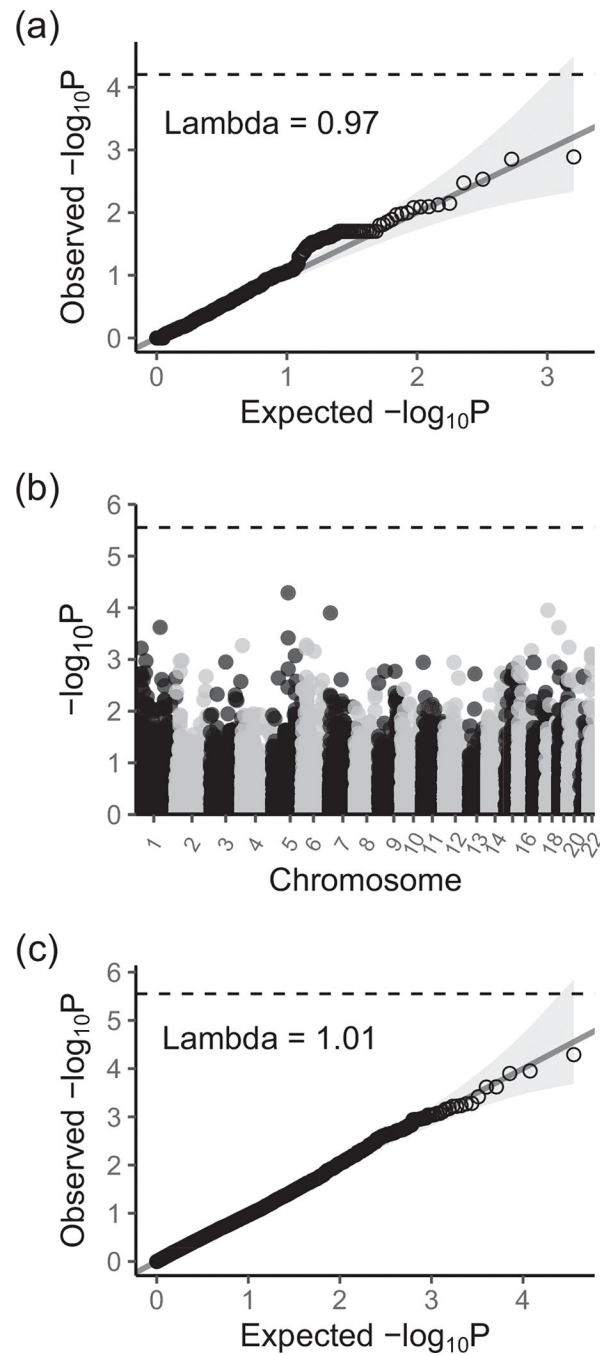


FIGURE 2 |

Split-hand/foot malformation quantile-quantile and Manhattan plots from case-control analyses of rare and common variants, National Birth Defects Prevention Study, 1997–2011. Quantile-quantile plots from (a) optimal unified sequence kernel-based association (SKAT-O) testing for rare, predicted damaging variants and (b) a Manhattan plot and (c) a quantile-quantile plot from logistic regression analysis of common variants. The horizontal line on each plot is the threshold for Bonferroni-corrected statistical significance.

TABLE 1 |

Characteristics of 26 cases with split-hand/foot malformation in the National Birth Defects Prevention Study, 1997–2011.

Characteristic	<i>n</i>	% ^a
Phenotype		
Cleft hand, unilateral	14	53.8
Cleft hand, bilateral	5	19.2
Cleft foot, unilateral or bilateral	4	15.4
Cleft hand and cleft foot, bilateral	3	11.5
Sex		
Male	16	61.5
Female	10	38.5
Maternal race/ethnicity (self-reported)		
Non-Hispanic White	19	73.1
Other	7	26.9
Paternal race/ethnicity (mother-reported)		
Non-Hispanic White	20	76.9
Other	6	23.1

^aPercentages may not sum to 100% because of rounding.

Validation of single nucleotide variants and small deletions in case children with split-hand/foot malformation in the National Birth Defects Prevention Study, 1997–2011.

TABLE 2 |

Case family ID	Gene	Variant	Variant type	dbSNP ID	Alternate allele frequency ^a	Inheritance pattern/ Zygosity	Role of gene in development or association of gene with birth defects
5	<i>SPOP</i>	ENST000000347630.2:c.1064C > T(p.Ser355Phe)	Missense	—	0	<i>De novo</i> / heterozygous	<i>SPOP</i> regulates turnover of Gli family transcription factors, components of the sonic hedgehog signaling pathway that is important in embryonic patterning (Schwend et al. 2013); <i>SPOP</i> loss leads to brachydactyly and osteopenia in mice (Cai and Liu 2016)
11	<i>FGF13</i>	ENST000000370603.3:c.69del(p.Gly24AlafsTer18)	Frameshift deletion	rs1275888012	1.9 × 10 ⁻⁵	X-linked recessive/ hemizygous	<i>Fgf13</i> regulates embryonic neural differentiation and limb patterning (Munoz-Sanjuan et al. 1999; Nishimoto and Nishida 2007); <i>FGF13</i> disruption in humans causes Genetic Epilepsy and Febrile Seizures Plus disorder (Puranam et al. 2015)
12	<i>ILF3</i>	ENST000000250241.8:c.333G > T(p.Glu111Asp)	Missense	rs755638784	1.4 × 10 ⁻⁵	Autosomal recessive/ compound heterozygous	<i>Ilf3</i> regulates embryonic skeletal muscle development, and loss of <i>Ilf3</i> in mice leads to defects in myocyte differentiation, resulting in disorganization of muscle fibers and muscle weakness (Shi et al. 2005)
12	<i>ILF3</i>	ENST000000250241.8:c.1936G > A(p.Gly646Arg)	Missense	rs758846034	3.1 × 10 ⁻⁵	Autosomal recessive/ compound heterozygous	
25	<i>UBA2</i>	ENST000000246548.4:c.177_180del(p.Arg59SerfsTer38)	Frameshift deletion	—	0	<i>De novo</i> / heterozygous	<i>Uba2</i> transient inhibition in pregnant mice resulted in offspring with craniofacial defects, congenital hydrocephalus, syndactyly, and blindness (Mata-Garrido et al. 2025)
26	<i>RBM10</i>	ENST000000377604.3:c.322C > T(p.Arg108Trp)	Missense	rs1489483300	5.0 × 10 ⁻⁶	X-linked recessive/ hemizygous	<i>RBM10</i> loss-of-function variants in humans are associated with brain malformations, cleft palate and other facial dysmorphism, limb defects, and abnormalities of the cardiac, gastrointestinal, genital, and renal systems (Kumps et al. 2021)

Abbreviations: dbSNP, National Center for Biotechnology Information database of Single Nucleotide Polymorphisms; *FGF13*, fibroblast growth factor 13; *ILF3*, interleukin enhancer binding factor 3; *RBM10*, RNA binding motif protein 10; *SPOP*, speckle type BTB/POZ protein; *UBA2*, ubiquitin like modifier activating enzyme 2.

^a Alternate allele frequency from gnomAD v4.1.0 database.

TABLE 3 |
Droplet digital PCR assay results for two predicted copy-number variants in split-hand/foot malformation family trios in the National Birth Defects Prevention Study, 1997–2011.

Copy-number variant location ^a (genes overlapped by region)	Variant size, based on exome sequencing data (kilobases)	Type of variation	Genomic locus targeted by probe ^a	Case family ID	Sample	Mean ± SD target gene:RNaseP ratio ^b
chr10:103190101–103436193 (<i>BTRC</i> , <i>DPCD</i> , <i>POLL</i> , <i>FBXW4</i>)	246.1	Duplication	chr10:103285832	8	Control DNA	0.943 ± 0.061
					Mother	0.903 ± 0.041
					Father	0.917 ± 0.034
					Child	1.187 ± 0.055
chr19:16552702–16590085 (<i>CALR3</i> , <i>EPS15L1</i>)	37.4	Duplication	chr19:16568651	9	Control DNA	1.010 ± 0.070
					Mother	1.396 ± 0.003 ^c
					Father	0.929 ± 0.023
				9	Child	0.798 ± 0.005

Abbreviations: *BTRC*, beta-transducin repeat containing E3 ubiquitin protein ligase; *CALR3*, calreticulin 3; *DPCD*, deleted in primary ciliary dyskinesia homolog (mouse); *EPS15L1*, epidermal growth factor receptor pathway substrate 15 like 1; *FBXW4*, F-box and WD repeat domain containing 4; *POLL*, DNA polymerase lambda; PCR, polymerase chain reaction.

^aLocation in GRCh37/hg19 coordinates.

^bValues were calculated from three replicate assays of sample DNA at a concentration of 1 ng/μL.

^cValue was calculated from only two replicate assays because the third assay failed and was excluded from the analysis.

TABLE 4 |
Split-hand/foot malformation case-control analysis results for rare and common variants, the National Birth Defects Prevention Study, 1997–2011.

Characteristic	Rare, predicted damaging variants	Common variants
Type of analysis ^a	SKAT-O	Logistic regression
Number of cases	26	26
Number of controls	1040	1191
Number of variants	11,710 variants in 799 genes	17,884 variants
Genomic inflation factor	0.97	1.01
Top hit	<i>ELMOD3</i> gene	ENST00000296754.3:c.166G > A(p.Glu561Lys) variant in <i>ERAP1</i>
Original <i>p</i> -value of top hit	1.29 × 10 ⁻³	5.12 × 10 ⁻⁵
Adjusted <i>p</i> -value of top hit ^b	0.46	0.71

Abbreviations: *ELMOD3*, ELMO domain containing 3; *ERAP1*, endoplasmic reticulum aminopeptidase 1; SKAT-O, optimal unified sequencing kernel association test.

^a All analyses were adjusted for sex and the first three principal components.

^b Benjamini-Hochberg method used to control the false discovery rate at 0.05.