High resolution microarray studies using the Affymetrix SNP 6.0 array identified duplications of chromosome 17p13.3 in individuals with Split Hand Foot Malformation with Long Bone Deficiency (SHFLD3)

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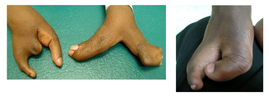
INTRODUCTION

Congenital limb malformations characterized by median clefts of the hands and/or feet comprise the primary clinical findings in Split-Hand/Foot Malformation (SHFM). SHFM presents with underdevelopment or absence of central digital rays and metacarpal/metatarsal bones and syndactyly of the remaining digits. Significant genetic heterogeneity has been reported with different syndromic and isolated forms of SHFM. Most forms of SHFM are autosomal dominant disorders with incomplete penetrance and variable expressivity. Early literature reports identified chromosomal aberrations in individuals with these disorders; however, reports have found single gene mutations as being causative of these malformations. Multiple families with SHFM1 and SHFM3 have been followed and studied at the Greenwood Genetic Center to identify deletions, and inversions involving 7q21q22, and duplications involving 10q24 for the SHFM loci. Microarray, chromosome analysis and different molecular methods (such as FISH, qPCR, etc) may be helpful for screening cases of SHFM/SHFLD of unknown etiology and aid in the identification of genomic loci associated with these malformations.

SPLIT-HAND/FOOT MALFORMATION (ECTRODACTYLY)



- Affects 1 in 8,000-25,000 individuals
- Deficiency of central rays of hands and feet,
- Median clefts of hands and feet, syndactyly of remaining digits
- Underdevelopment/absence of phalanges, metacarpal bones, and metatarsal bones

Disorders	Location	Implicated Genes
Isolated SHFM		
SHFM1	7q21q22	*SHFM1, DLX5, DLX6*
SHFM2	Xq26	*FGF13, TONDU*
SHFM3	10q24	*FBXW4*
SHFM4	3q27	*TP63*
SHFM5	2q31	*HOXD, DLX1, DLX2*
SHFM6	12q13	*WNT10B*
EEC and related syndromes		
Ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC) syndrome	3q27	*TP63*
Limb-mammary syndrome (LMS)	3q27	*TP63*
Other selected SHFM syndromes		
Ectrodactyly-sensorineural hearing loss	7q21	*DLX5, DLX6, DSS1*
Microcephaly-microphthalmia-ectrodactyly-prognathism (MMEP)	6q21	*SNX3*
Split hand/foot malformation-long bone deficiency (SHFLD1)	1q42.2q43	
Split hand/foot malformation-long bone deficiency (SHFLD2)	6q14.1	
Split hand/foot malformation-long bone deficiency (SHFLD3)	**17p13.3**	***ABR, BHLHA9***

MATERIALS AND METHODS

Clinical Specimens: Peripheral blood was collected and processed for cytogenetic analysis and molecular studies. The clinical features and pedigrees of the patients were assessed.

DNA isolation: DNA was isolated from peripheral blood using Qiagen DNA minikit (Qiagen, Valencia, CA). The quality and quantity of the isolated DNA was assessed by agarose gel electrophoresis and spectrophotometric analysis, respectively.

SNP Microarray: Microarray was performed on the Affymetrix 6.0 platform according to manufacturer's instructions (Affymetrix, Santa Clara, CA). Genomic DNA was isolated and purified using the QIAamp DNA blood mini kit (Qiagen, California). DNA concentration and purity were determined with a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Briefly, 250 ng DNA was digested with Nsp I or Sty I (New England Biolabs) and ligated to the appropriate adaptors for subsequent PCR amplification (30 cycles; Titanium DNA Amplification Kit; Clontech, #639240). The PCR products were purified using Agencourt AMPure Magnetic Beads (Fisher, # NC9113390), fragmented, labeled and added to the array chips. Hybridization was performed for 16 to 18 hours in the GeneChip® Hybridization Oven 645. The arrays were washed and stained in the GeneChip® Fluidics Station 450 and scanned using Affymetrix Genome wide SNP 6.0 array protocol (California, US) using the GeneChip® Scanner 3000 7G. Copy number analysis was performed with Affymetrix's Genotyping Console 4.0 using the *in silico* control of 270 HapMap samples.

Quantitative PCR analysis (qPCR): qPCR was performed for confirmation of the copy gain at 17p13.3 and screening of the additional cohort of patients with SHFM/SHFLD and their family members. The EP RealPlex4 Mastercycler (Eppendorf AG, Hamburg, Germany) was employed to determine the relative quantitation of genomic dosage by the relative threshold cycle (ΔΔCT) method (Livak, KJ., 2001). Briefly, the genomic DNA template was used to generate PCR amplicons in triplicate for each individual and three to five controls in the assay. Genomic dosage for the *ABR* and *BHLHA9* gene was determined by SYBR green incorporation using Taqman RNAseP reference for each sample (ABI, Carlsbad, CA). Several sets of primer pairs within the region of duplication on 17p13.3 were designed and validated to check the efficiency of each primer pair. The best primer pair from each region was chosen based on sequences that were predicted to be both amenable to qPCR and informative for the purpose of confirming the microarray results, as shown in Figure 3. Relative genomic dosage was calculated as $2^{-\Delta\Delta CT}$ where ΔCt = (mean Ct_{Target}) – (mean $Ct_{Reference}$) and $\Delta\Delta CT = \Delta Ct_{patient} - \Delta Ct_{control}$. Analyses were done for all the individuals and the copy gains were scored relative to normal controls.

SPLIT HAND FOOT MALFORMATION WITH LONG BONE DEFICIENCY

- Same hand/foot findings as SHFM with underdeveloped/absent tibia or other long bones, extremely variable expressivity
- Autosomal dominant with incomplete penetrance

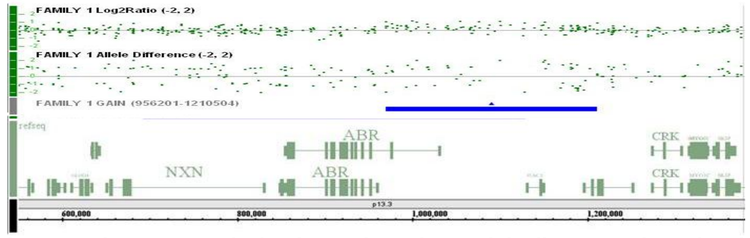


Figure 1. Microarray analysis of one of our three originally reported unrelated families with SHFLD and distinct duplications of 17p13.3.

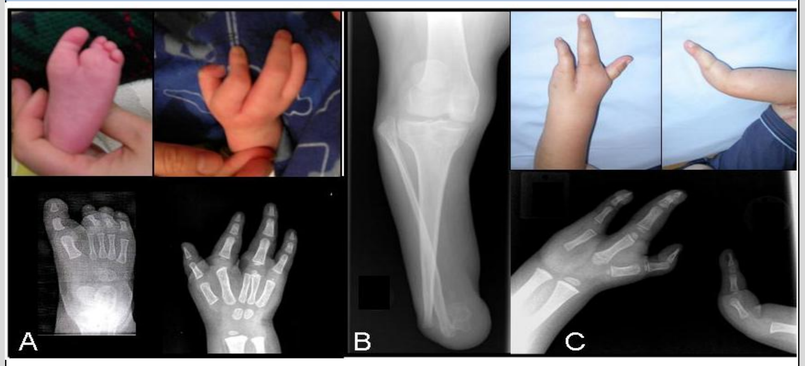


Figure 2. Clinical findings in our 3 originally reported families with dup 17p13.3. (A) Hand and foot findings, (B) Tibial hypoplasia, (C) Monodactyly and oligodactyly with split hand [*Eur J Hum Genet* 19(11):1144-51].

SCREENING OF 30 SHFM/SHFLD CASES FOR DUPLICATION OF 17p13.3

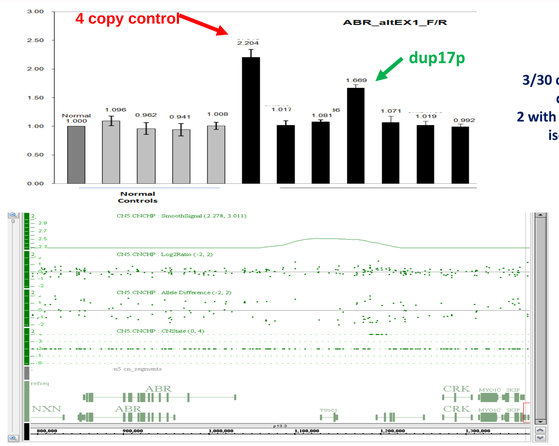


Figure 3 (Top panel): Representative qPCR results from cohort of 30 cases of SHFM or SHFLD using primer pairs for *ABR* altEX1_F/R. Normal controls (3-5) were included on each run along with a 4x positive control. The genomic dosage of the patients (black) relative to normal controls (light grey) is shown with the expected normal value indicated (dark grey). (Lower Panel): Representative array result from one of our new duplication 17 cases.

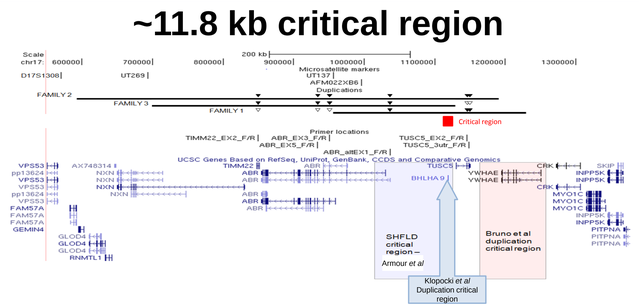


Figure 4. Modified figure from Armour *et al.* 2011. Shown in red is the redefined critical region based on the three new duplication 17 cases identified in our cohort of SHFM and SHFLD patients. Duplication of this critical region may cause SHFM and/or SHFLD through increased dosage of *BHLHA9*. Klopocki *et al.* recently reduced the size of the duplication critical region to include only *BHLHA9* which encodes a basic helix-loop-helix protein and exhibits a high degree of conservation across species. Alternatively, it could alter the dosage of a regulatory element involved in limb development or disrupt the interaction between a nearby regulatory element and its target gene(s).

FISH OF DUPLICATION OF CHROMOSOME 17p13.3

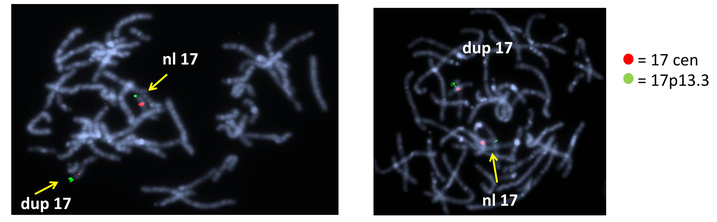
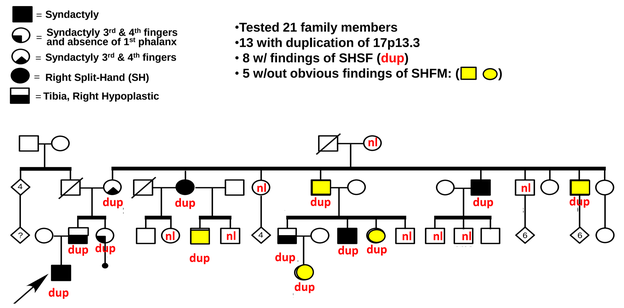


Figure 5. Representative FISH images of patient with duplication. DNA from fosmid clone WI2-2179K12 (G248P87194F6) was labeled with digoxigenin and visualized using anti-digoxigenin on a Zeiss Axioplan microscope. The probe was 40.5 kb in size and is between *NXN and ABR*. Digital photographs were taken with Leica (Applied Imaging) Probevision software.

RESULTS IN FOUR- GENERATION FAMILY WITH SHFLD3



An extended molecular analysis of 21 family members from 4 generations of one of the familial cases revealed 8 affected family members harboring the 17p13.3 duplication (including 2 with SHFLD and 6 with only hand/foot involvement). However, 5 individuals found to be carrying the duplication did not have a significant clinical phenotype associated with SHFM or SHFLD3 documenting incomplete penetrance associated with this disorder.

CONCLUSIONS

1. **17p13.3 duplications causing SHFM and SHFLD phenotypes have highly variable expressivity.**
2. **Our studies have shown that duplications of 17p13.3 are a relatively frequent cause of SHFM and SHFLD phenotypes.**
3. **10% of our cases of SHFM or SHFLD revealed a duplication of chromosome 17p13.3 by either Affymetrix's High Resolution SNP Microarray or custom qPCR studies.**
4. **FISH studies performed using a fosmid clone showed that the duplication was tandem in one patient.**
5. **Duplications of 17p13.3 should be investigated in all cases of SHFLD and other SHFM cases of unknown etiology.**
6. **Incomplete penetrance should be considered in the context of genetic counseling and molecular testing for unaffected at-risk family members.**
7. **The precise molecular mechanisms causing the SHFM/SHFLD phenotype at this locus have not yet been determined. However, the role and contribution of possible gene modifiers at either a nearby locus or a different region of the genome may help in elucidating the molecular mechanism in these disorders.**

ACKNOWLEDGEMENTS AND REFERENCES

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References:

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Klopocki, E. *et al.* (2012) Duplications of *BHLHA9* are associated with ectrodactyly and tibia hemimelia inherited in non-Mendelian fashion. *J Med Genet* 49:119-125. doi:10.1136/jmedgenet-2011-100409.