

Genomewide Linkage Scan for Split-Hand/Foot Malformation with Long-Bone Deficiency in a Large Arab Family Identifies Two Novel Susceptibility Loci on Chromosomes 1q42.2-q43 and 6q14.1

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Split-hand/foot malformation with long-bone deficiency (SHFLD) is a rare, severe limb deformity characterized by tibia aplasia with or without split-hand/split-foot deformity. Identification of genetic susceptibility loci for SHFLD has been unsuccessful because of its rare incidence, variable phenotypic expression and associated anomalies, and uncertain inheritance pattern. SHFLD is usually inherited as an autosomal dominant trait with reduced penetrance, although recessive inheritance has also been postulated. We conducted a genomewide linkage analysis, using a 10K SNP array in a large consanguineous family (UR078) from the United Arab Emirates (UAE) who had disease transmission consistent with an autosomal dominant inheritance pattern. The study identified two novel SHFLD susceptibility loci at 1q42.2-q43 (nonparametric linkage [NPL] 9.8, $P = .000065$) and 6q14.1 (NPL 7.12, $P = .000897$). These results were also supported by multipoint parametric linkage analysis. Maximum multipoint LOD scores of 3.20 and 3.78 were detected for genomic locations 1q42.2-43 and 6q14.1, respectively, with the use of an autosomal dominant mode of inheritance with reduced penetrance. Haplotype analysis with informative crossovers enabled mapping of the SHFLD loci to a region of ~18.38 cM (8.4 Mb) between single-nucleotide polymorphisms *rs1124110* and *rs535043* on 1q42.2-q43 and to a region of ~1.96 cM (4.1 Mb) between *rs623155* and *rs1547251* on 6q14.1. The study identified two novel loci for the SHFLD phenotype in this UAE family.

Split-hand/foot malformation with long-bone deficiency (SHFLD [MIM %119100]), a rare and severe limb deformity, is also known as “cleft hand and absent tibia,” “aplasia of tibia with ectrodactyly,” “ectrodactyly with aplasia of long bones,” or “tibial aplasia (TA) with split-hand/split-foot deformity.” The clinical manifestations are highly variable and range from virtually no malformation to ectrodactyly and tibial hypoplasia or aplasia with or without associated anomalies.^{1–3} The incidence of SHFLD has been estimated to be ~1 per million live births.⁴ It is characterized by hypoplasia or aplasia of tibia, with relatively intact fibula, associated with split-hand/split-foot deformity that more often affects the upper limb. SHFLD was first described in 1575,^{5,6} and Otto² also reported an affected fetus. Families with SHFLD have been reported with autosomal dominant, recessive, and sporadic forms of inheritance.^{1–3,7–18} A number of malformations have been described in association with SHFLD, including triphalangeal thumbs and polydactyly,⁸ cleft lip/palate,¹⁶ cardiac defects,¹⁹ vaginal agenesis,²⁰ cardiovascular defects,²¹ hypohidrotic ectodermal dysplasia,²² and ectrodactyly.²³ We present genomewide linkage analysis of one large multi-

generational Arab family with SHFLD, using the GeneChip Mapping EA 10K Array (Affymetrix) containing ~10,000 SNP markers. The present analysis provided significant evidence for two susceptibility loci, one on a genomic region spanning 8.4 Mb on chromosome 1q42.13-q43 and another on a region of 4.1 Mb on 6q14.1. We hypothesize that SHFLD could fit the model of digenic inheritance.^{24–26}

Material and Methods

We recently reported a large multigenerational consanguineous family (UR078) from the United Arab Emirates (UAE) with autosomal dominant SHFLD.²³ The original eight-generation pedigree (fig. 1A) with 10 consanguineous marriages is much larger than the present partial pedigree used for gene-mapping analysis (fig. 1B). The majority of the family members live in the UAE; however, a few reside in Oman. Of the 145 individuals in this family, 23 (14 males and 9 females) showed an abnormal phenotype, ranging from mild to severe defects involving upper and lower limbs (fig. 1C). The nine affected individuals included in this linkage study all had severe TA, some with additional findings of split hand/foot, syndactyly of fingers/toes, hypoplastic big toes, absence of middle phalanges of some toes, hypoplastic tibiae, and

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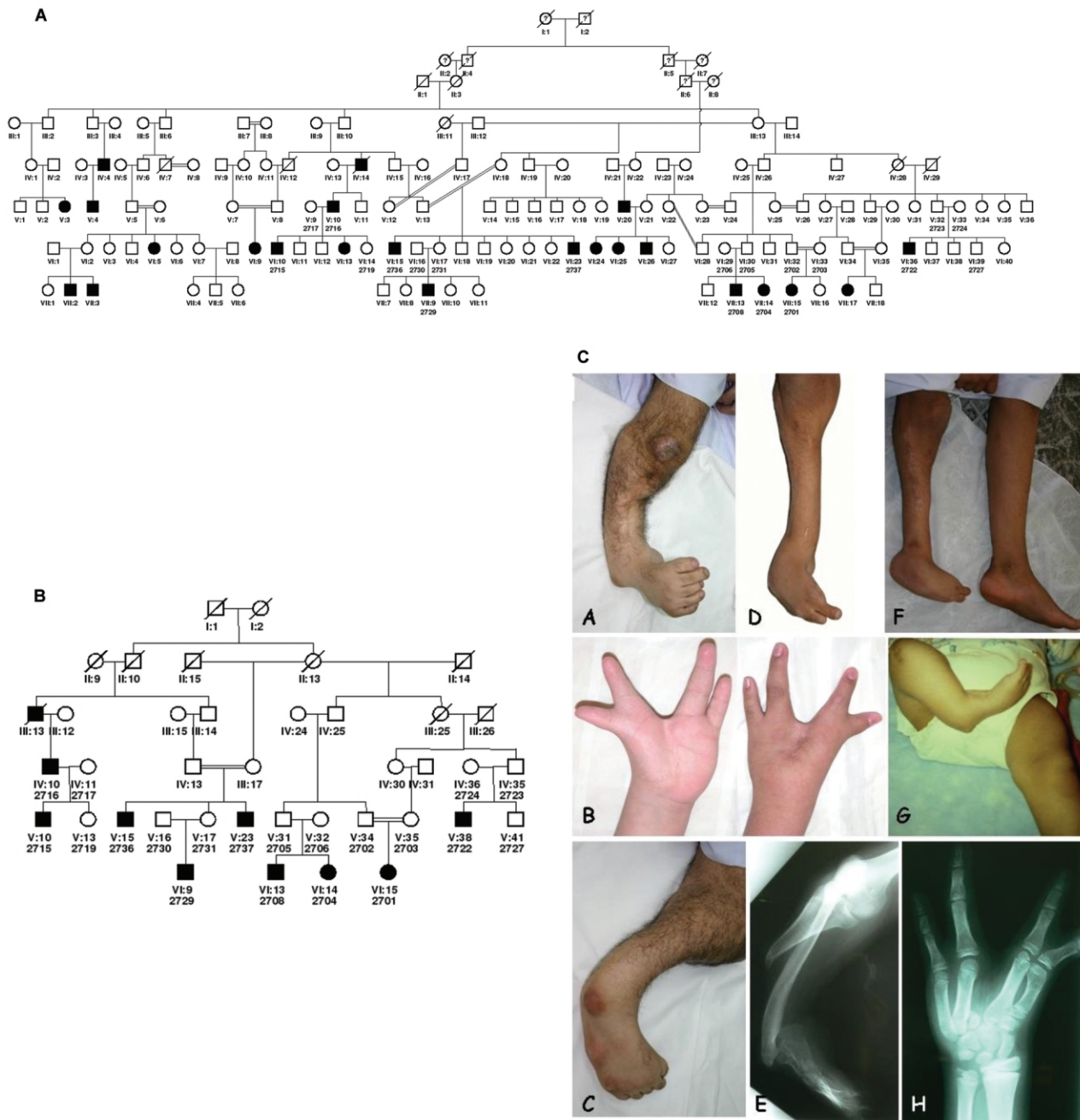


Figure 1. Complete (A) and partial (B) pedigrees of family UR078 with SHFLD. Affected individuals are shown with blackened symbols, and unaffected individuals are shown with unblackened symbols. Individuals used in the linkage analysis are numbered under their symbols in the pedigree. C, Clinical and x-ray photos of selected individuals (A–H) from family UR078 with SHFLD.

beaked nose. No other abnormalities—in particular, no cleft lip/palate or ectodermal dysplasia—were observed in this family.

Blood samples were collected from all cooperative and informative family members after informed consent for the genetic studies. DNA was not collected from individuals in the pedigree who could not be reliably defined as “affected,” because they had minimal symptoms, such as short big toe or syndactyly without TA. DNA from peripheral blood samples was isolated using standard procedures (Genra Kit).

A genomewide search was undertaken using a GeneChip Mapping 10K XbaI Array containing 10,555 SNPs. These SNP markers are equally distributed in the genome, with a mean intermarker distance of 210 kb and an average heterozygosity of 0.38 (Affymetrix). The assay was done using 250 ng of genomic DNA, and >99% of the SNPs were determined unequivocally for each sample. Scanned images were processed with Affymetrix Micro Array Suite software. Data were analyzed with GDAS v2 software. PedCheck was used for detection of Mendelian errors.²⁷

Table 1. Initial Genomewide Linkage Analysis Results Using NPL ($P < .01$), Revealing Six Genomic Regions that May Harbor the Putative SHFLD Susceptibility Loci

SNP Marker	Position		Nonparametric Analysis	
	Cytogenetic	Build 35.1	NPL	P
<i>rs951908</i>	1p36.13	19372422	6.20	.004000
<i>rs530157</i>	1q31.1	183800525	5.58	.008400
<i>rs1405633</i>	1q42.3	234486121	9.79	.000065
<i>rs720096</i>	4q34.3	179788065	5.63	.006900
<i>rs723142</i>	6q14.1	83094274	7.09	.001200
<i>rs2040847</i>	17p13.1	6498735	5.01	.009200

SNP genotype data were imported into the linkage analysis programs GENEHUNTER²⁸ and MERLIN.²⁹ Since the parameters of the disease model were uncertain, in the initial genome scan we assessed the evidence of linkage with nonparametric, penetrance-independent, affected-only, and allele-sharing models. Owing to the size of the family being studied, the SNP data were initially analyzed by splitting the entire family into two separate families (UR078A and UR078B), with minimal overlap between them. On finding significant evidence of linkage by exceeding the predetermined threshold ($P < .01$), we performed two-point, as well as multipoint (four-point), LOD scores maximized over various plausible genetic model parameters (MOD-score analysis) on the entire pedigree, using the LINKAGE analysis package. For each marker, we assessed our linkages with the white and Asian allele frequencies provided by Affymetrix. Linkage analysis using Asian or white allele frequencies for the Arab population may not be appropriate, and it may impact the parametric linkage results. However, since we have only a few founders available from this family, we could not estimate the marker-allele frequencies from the family data. The map order and intermarker distances between SNPs were based on the National Center for Biotechnology Information (NCBI) build 35.1.

To assess the false-positive evidence of linkage, we performed a simulation experiment to evaluate our results, using empirical P values. The simulations were designed to match our observed data in marker density, marker informativeness, pedigree struc-

ture, and individual phenotypes. We generated 10,000 replicate data sets, under the null hypothesis of no linkage, to estimate the empirical P value. Putative haplotypes containing the disease-causing loci were determined by using the critical recombinants across the family members.

It has been demonstrated that applying linkage analyses that assume linkage equilibrium to dense markers may lead to bias,^{30,31} especially when analyzing SNP linkage maps in data sets in which some parental genotypes are missing. Therefore, we assessed the impact of linkage disequilibrium (LD) on linkage at the linked regions. We used MERLIN to accommodate marker-marker LD in both parametric and nonparametric analyses, by organizing closely located adjacent markers into clusters. Although many empirical studies have shown that the extent and distribution of LD is extremely variable throughout the genome, in most cases significant LD does not influence markers separated by >0.1 cM in outbred populations.^{32–34} However, to be conservative, we used markers within 0.3 cM of one other in a cluster.

Results

Before this genomewide genotyping, we had excluded the published candidate genomic regions on chromosomes 7p13, 7q36, 8q24.1, and 10q24 by linkage and haplotype analysis.²³ Initial analysis with GENEHUNTER revealed six genomic regions ($P < .01$) on chromosomes 1p36, 1q31, 1q42, 4q34, 6q14, and 17p13 that may harbor the putative SHFLD susceptibility loci (table 1) (fig. 2). Subsequent analyses with MERLIN indicated similar results in these regions. Among these six linked regions, two linkages were found to be the most interesting—one on chromosome 1q42 and the other on 6q41. The maximum multipoint NPL yielded significant evidence (NPL 9.8, $P = .000065$) for SNP marker *rs966302* (physical map position 232,585,612 bp) on chromosome 1q42.13–q43 (table 2). The locus, which was identified on chromosome 6q14.1, yielded the second-highest NPL results (NPL 7.12, $P = .00089$) at marker *rs688867* (80,995,947 bp) (table 2). With the use of 10,000 simulations, the empirical P values for

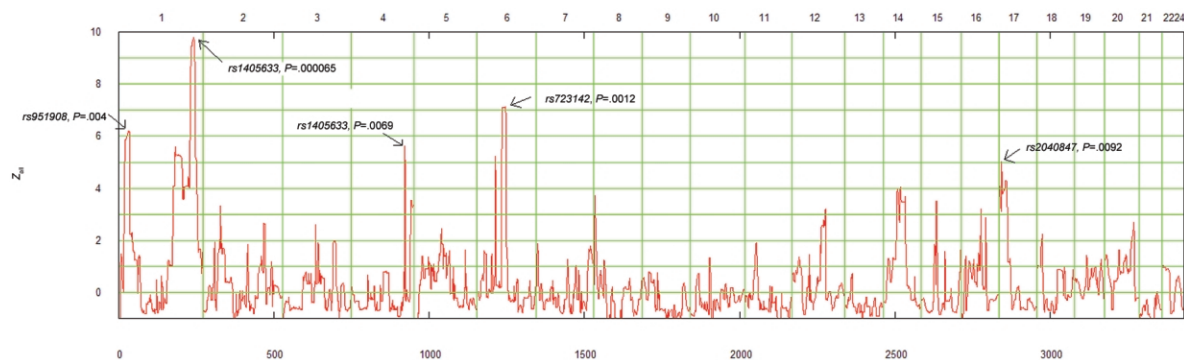


Figure 2. Multipoint linkage analysis using NPL in the genomewide scan for the Arab family with SHFLD. The X-axis represents the chromosome locations for all the autosomes, and the Y-axis represents the Z_{all} . P values are derived from the NPL_{all} statistics. The highest peak is on chromosome 1 with $P = .000065$ (NPL $Z = 9.8$), and the second highest is on chromosome 6 (NPL 7.12, $P = .00089$). Arrows indicate the SNP markers with the highest NPL peaks.

Table 2. SHFLD Loci in Family UR078 Mapped by Linkage Analysis to Chromosome 1q42.13-1q43 and 6q14.1 Regions

SNP Marker	Position		Physical (bp)	NPL Multipoint Linkage Genome Scan	
	Genetic (cM)	Cytogenetic		NPL	P
<i>rs1124110</i>	233.32	1q42.13	226,617,707	4.61	.009373
<i>rs1933633</i>	234.32	1q42.2	227,231,111	6.25	.003083
<i>rs953244</i>	235.28	1q42.2	228,130,318	7.76	.000215
<i>rs4333837</i>	235.37	1q42.2	228,331,909	7.84	.000201
<i>rs967433</i>	235.38	1q42.2	228,338,550	7.85	.000201
<i>rs720806</i>	237.54	1q42.2	228,945,997	8.94	.000097
<i>rs965917</i>	238.86	1q42.2	229,690,846	9.38	.000086
<i>rs1294330</i>	238.97	1q42.2	229,755,343	9.45	.000084
<i>rs780245</i>	239.42	1q42.2	230,004,265	9.48	.000084
<i>rs923975</i>	239.95	1q42.2	230,304,605	9.58	.000084
<i>rs923976</i>	239.95	1q42.2	230,304,802	9.58	.000084
<i>rs955612</i>	241.23	1q42.3	231,023,328	9.58	.000084
<i>rs1416473</i>	244.23	1q42.3	231,889,300	9.74	.000084
<i>rs966302</i>	245.54	1q42.3	232,585,612	9.79	.000065
<i>rs1405633</i>	246.04	1q42.3	232,745,539	9.79	.000065
<i>rs1749569</i>	246.04	1q42.3	232,745,622	9.79	.000065
<i>rs966364</i>	246.48	1q42.3	232,883,969	9.75	.000084
<i>rs959175</i>	247.01	1q43	233,078,923	9.71	.000084
<i>rs1337797</i>	248.33	1q43	233,625,390	9.44	.000084
<i>rs950964</i>	248.62	1q43	233,746,722	9.33	.000086
<i>rs1074189</i>	249.44	1q43	234,087,534	8.74	.000109
<i>rs1361358</i>	250.12	1q43	234,370,458	7.80	.000215
<i>rs535043</i>	251.70	1q43	235,022,077	5.23	.009182
<i>rs623155</i>	90.01	6q14.1	79,324,200	7.10	.000897
<i>rs1415863</i>	90.24	6q14.1	79,756,878	7.10	.000897
<i>rs1414280</i>	90.36	6q14.1	79,985,449	7.11	.000897
<i>rs721265</i>	90.63	6q14.1	80,549,653	7.10	.001173
<i>rs719172</i>	90.65	6q14.1	80,611,300	7.11	.000897
<i>rs688867</i>	90.78	6q14.1	80,995,947	7.12	.000897
<i>rs723587</i>	90.82	6q14.1	81,113,079	7.12	.000897
<i>rs1902066</i>	90.93	6q14.1	81,402,752	7.10	.000897
<i>rs1377986</i>	90.96	6q14.1	81,463,288	7.10	.001173
<i>rs1584896</i>	90.96	6q14.1	81,463,350	7.10	.001173
<i>rs1584897</i>	90.96	6q14.1	81,463,381	6.99	.001266
<i>rs724993</i>	91.08	6q14.1	81,799,004	7.02	.001217
<i>rs962984</i>	91.19	6q14.1	82,120,869	7.04	.001217
<i>rs962983</i>	91.19	6q14.1	82,121,184	7.04	.001217
<i>rs1343232</i>	91.21	6q14.1	82,187,051	7.04	.001207
<i>rs719144</i>	91.4	6q14.1	82,388,507	7.07	.001186
<i>rs733413</i>	91.6	6q14.1	82,587,633	7.09	.001173
<i>rs72968</i>	91.6	6q14.1	82,587,967	7.09	.001173
<i>rs1931621</i>	91.61	6q14.1	82,596,847	7.09	.001173
<i>rs1342196</i>	91.75	6q14.1	82,862,011	7.10	.001173
<i>rs2226121</i>	91.75	6q14.1	82,862,272	7.10	.001173
<i>rs1556778</i>	91.75	6q14.1	82,862,533	7.10	.001173
<i>rs950611</i>	91.83	6q14.1	83,023,894	7.10	.001173

NOTE.—Recombination narrowed the candidate region to an interval flanked by *rs1294330* and *rs1361358* for chromosome 1q and to an interval flanked by *rs623155* and *rs1547251* for chromosome 6q.

1q42-43 and 6q41 were .0004 and .006, respectively. These genomic regions were also supported by parametric linkage analysis that used the entire family. The multipoint (four-point) LOD scores for 1q42.13-43 and 6q41.1 loci were 3.20 and 3.78, respectively (table 3). The best-fitted model for the 1q42-43 linkage was incomplete dominance with 70% penetrance and disease-allele frequency 0.01,

and for 6q41 it was incomplete dominance with 50% penetrance and disease-allele frequency 0.0001. Evidence of linkage for the remaining candidate regions was not supported by the parametric linkage analyses.

In our initial linkage analysis, we used the Asian allele frequencies provided by Affymetrix, which may not truly represent the genetic makeup of the Arab population. To assess the impact of allele frequencies on our linkage findings, we used the white allele frequencies and recomputed the parametric linkage. Interestingly, we reproduced our initial linkage findings. The LOD score at 1q42.13-43 increased from 3.2 to 4.5, and at 6q41.1 the LOD score was reduced slightly, from 3.78 to 3.2. The multipoint linkage was reanalyzed to accommodate marker-marker LD in both nonparametric and parametric analyses, by the organization of closely located adjacent markers into clusters. Several clusters of two to six SNPs demonstrated LD. With the assumption of no LD within the cluster, MERLIN uses population haplotype frequencies while calculating linkage. The NPL scores at 1q42.13-43 and 6q41 were reduced from 9.8 to 8.5 and from 7.12 to 5.15, respectively. However, this reduction of linkage scores might be due to both the effect of LD and the reduction of information content (IC). Because of the clustering (hence, the reduction of the number of markers), the IC was reduced from 89% to 74% at the peak region at 1q42 and from 80% to 69% at 6q14.1. Nonetheless, evidence of linkage at both peaks is consistent.

Haplotype analysis was constructed using 28 informative SNP markers on 1q42.13-q43 and revealed informative recombination events in affected individual 2729, which confined the SHFLD candidate region to ~18.38 cM (8.4 Mb) between SNPs *rs1124110* (226,617,707 bp) and *rs535043* (235,022,077 bp). Similar haplotype analysis and critical recombination events across the affected family members (except 2722) on chromosome 6q14.1 narrowed the genomic region to ~1.96 cM (4.1 Mb). The area is bordered by proximal marker *rs623155* (79,324,200 bp) and distal marker *rs1547251* (83,462,826 bp) (fig. 3).

Discussion

We identified novel genomic regions on 1q42.13-q43 and 6q14.1 that harbor high-risk variants for SHFLD in this UAE family. The 8.4-Mb genomic interval on 1q42.13-q43 contains 17 known putative transcripts, whereas the 4.1-Mb genomic region on chromosome 6q14.1 contains six annotated transcripts (Ensembl). Logical candidate genes include homeobox-like protein 1 (*MIXL1* [MIM 609852]), ectodysplasin A receptor-associated death domain (*EDAR-ADD* [MIM 606603]), a human galectin-8-related gene (*LGALS8* [MIM 606099]), alpha-actinin gene (*ACTN2* [MIM 102573]), protein related to differential screening-selected gene aberrative in neuroblastoma and cerberus (*GREM2* [MIM 608832]), and choroideremia-like (*CHML* [MIM 118825]). There is no previous evidence of linkage that would indicate that 1q or 6q regions are involved in

Table 3. Multipoint Linkage Data for Markers on Chromosomes 1q42.13-1q43 and 6q14.1

SNP Marker Order	Four-Point Analysis (Recombination Fraction)						
	0	.01	.05	.1	.2	.3	.4
1q42.2-q43:							
<i>rs1749569-rs966364-rs959175</i>	-1.40	-.57	.03	.23	.26	.17	.07
<i>rs966364-rs959175-rs1337797</i>	1.70	1.66	1.50	1.30	.91	.54	.24
<i>rs959175-rs1337797-rs950964</i>	2.45	2.39	2.14	1.84	1.23	.68	.26
<i>rs1337797-rs950964-rs1074189</i>	3.20	3.10	2.80	2.40	1.60	.90	.40
<i>rs950964-rs1074189-rs1361358</i>	2.70	2.70	2.40	2.00	1.30	.70	.30
<i>rs1074189-rs1361358-rs535043</i>	.40	.50	.60	.60	.40	.20	.00
6q14.1:							
<i>rs1414280-rs721265-rs719172</i>	1.26	1.72	2.02	1.95	1.52	.96	.41
<i>rs721265-rs719172-rs688867</i>	.10	1.80	2.20	2.00	1.50	.90	.40
<i>rs719172-rs688867-rs723587</i>	3.78	3.70	3.38	2.96	2.10	1.25	.51
<i>rs688867-rs723587-rs1902066</i>	3.38	3.31	3.00	2.61	1.83	1.08	.45
<i>rs723587-rs1902066-rs1377986</i>	1.54	1.50	1.35	1.15	.78	.46	.19
<i>rs1902066-rs1377986-rs1584896</i>	1.93	1.89	1.69	1.46	.99	.56	.22
<i>rs1342196-rs2226121-rs1556778</i>	3.17	3.10	2.83	2.48	1.77	1.08	.47

NOTE.—Parametric linkage analysis using whole-family data.

either TA or split-hand/foot malformation; on the other hand, 3q27, 7q21, 10q24, and Xq26³⁵⁻³⁸ have been implicated in familial split-hand/foot malformation. Interestingly, the 1q42-qter region harbors genetic variation related to several developmental phenotypes. Distal trisomy 1q³⁹⁻⁴¹ or subtelomeric 1qter deletion⁴² are associated with multiple developmental anomalies, including malformed fingers and toes.

Inter- and intrafamilial variability is common in families with SHFLD anomalies.^{2,3,12,23,43,44} Autosomal dominant inheritance with reduced penetrance was reported by Marioni et al.¹⁰ In the present family, individuals 2702, 2705, 2723, and 2731, who had apparently normal phenotypes, were parents of affected children who carried only affected haplotypes for both linked regions. All parents who had affected children shared affected haplotypes from both chromosomes 1 and 6. Therefore, we speculate that individual 2727, with normal phenotype and affected genotypes from both the chromosomes, may produce children with affected phenotypes. The haplotype data also supported the autosomal dominant mode of inheritance in this family. None of the unaffected spouses analyzed (i.e., 2717, 2706, 2703, and 2724) showed affected genotypes for 1q42-43 and 6q14.1 markers. An affected individual (2722), his unaffected brother (2727), and his phenotypically unaffected parent (2723), all of whom carried affected haplotypes for chromosome 1q, also shared a small portion of affected haplotypes for chromosome 6q (i.e., 0.56 cM), which was bordered by SNP markers *rs721265* and *rs962984*. We reconstructed haplotypes for chromosome 6q markers of individuals whose DNA was unavailable and found that the small portion of affected haplotypes in these individuals was transmitted from an apparently unrelated grandparent. It is possible that this grandparent may be indeed related to this family, since consanguinity is common in Arab communities. More-

over, the observed 10 consanguineous marriages suggest the possibility of pseudodominance in this family due to the high frequency of mutant alleles. The reported rate of consanguinity in the UAE population exceeds 50%.⁴⁵

The data from the linkage analysis indicate that more than one locus contributes to SHFLD. The present family (UR078) provided significant linkage at chromosome 1q42.13-q43 and strong evidence of linkage at 6q14.1. We thus hypothesize that the phenotype in this family could be due to digenic inheritance²⁴; however, it is difficult to prove this hypothesis until we identify the pathologic mutations. The hypothesis of digenic inheritance is supported by a detailed analysis of the haplotypes and the segregating phenotype of all the family members. For example, the phenotypically unaffected 10-year-old individual (2719) inherited the disease-linked haplotype from her affected father only on chromosome 6, whereas, for chromosome 1, the normal haplotype was inherited. A crossover event in this individual (V-13) on chromosome 6 (*rs1529992/rs763672*) further reduced the proximal risk haplotype. However, phenotypically unaffected individuals should not be used for defining the susceptibility region, since SHFLD has demonstrated incomplete penetrance.

Phenotypically unaffected or affected parents who have affected haplotypes produced affected children who have affected haplotypes from chromosomes 1 and 6. This indicates that the loci on both chromosomes are essential

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 3. Genotypes and haplotypes of chromosomes 1q and 6q. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

for the phenotypic expression. We also, however, observed individuals with no manifestation of the phenotype (i.e., 2702, 2705, 2723, 2727, and 2731) who carry the two disease-related haplotypes. Reduced penetrance at each locus is a possible explanation for this normal phenotype; alternatively, additional modifier loci may be required for full disease expression. It is also possible that one of these linked loci is a major dominant determinant and that the other is a modifier genomic variant. Similar suggestions were made by Zlotogora²⁴ with reference to the split-hand/split-foot-malformation phenotype. The present study provides evidence of SHFLD susceptibility loci on 1q42.13-q43 and 6q14.1. Further studies are needed to delineate the role of other potential loci involved in SHFLD in the families of different geographic origins. However, the statistically significant evidence of the linkage to 1q and 6q is of interest and should facilitate efforts to identify the underlying susceptibility genes.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Affymetrix, <http://www.affymetrix.com/products/arrays/specific/10k.affx>
 Ensembl, <http://www.ensembl.org/>
 MERLIN, <http://www.sph.umich.edu/csg/abecasis/Merlin/>
 NCBI, <http://www.ncbi.nlm.nih.gov/> (for build 35.1)
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for SHFLD, *MIXL1*, *EDARADD*, *LGALS8*, *ACTN2*, *GREM2*, and *CHML*)

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