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Evaluation of Genes Involved in Limb Development, Angiogenesis, and Coagulation as Risk Factors for Congenital Limb Deficiencies

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Abstract

We conducted a population-based case-control study of single nucleotide polymorphisms (SNPs) in selected genes to find common variants that play a role in the etiology of limb deficiencies (LD)s. Included in the study were 389 infants with LDs of unknown cause and 980 unaffected controls selected from all births in New York State (NYS) for the years 1998 to 2005. We used cases identified from the NYS Department of Health (DOH) Congenital Malformations Registry. Genotypes were obtained for 132 SNPs in genes involved in limb development (SHH, WNT7A, FGF4, FGF8, FGF10, TBX3, TBX5, SALL4, GREM1, GDF5, CTNNB1, EN1, CYP26A1, CYP26B1), angiogenesis (*VEGFA, HIF1A, NOS3*), and coagulation (*F2, F5, MTHFR*). Genotype call rates were >97% and SNPs were tested for departure from Hardy-Weinberg expectations by race/ethnic subgroups. For each SNP, odds ratios (OR)s and confidence intervals (CI)s were estimated and corrected for multiple comparisons for all LDs combined and for LD subtypes. Among non-Hispanic white infants, associations between *FGF10* SNPs rs10805683 and rs13170645 and all LDs combined were statistically significant following correction for multiple testing (OR=1.99; 95% CI=1.43-2.77; uncorrected p=0.000043 for rs10805683 heterozygous genotype, and OR=2.37; 95% CI=1.48-3.78; uncorrected p=0.00032 for rs13170645 homozygous minor genotype). We also observed suggestive evidence for associations with SNPs in other genes including *CYP26B1* and *WNT7A*. Animal studies have shown that *FGF10* induces formation of

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the apical ectodermal ridge and is necessary for limb development. Our data suggest that common variants in *FGF10* increase the risk for a wide range of non-syndromic limb deficiencies.

Keywords

limb deficiencies; polymorphisms; FGF10

INTRODUCTION

Limb deficiencies (LD) occur in approximately 8/10,000 births [Gold et al., 2011; Stoll et al., 2010]. Although mechanical disruption (chorionic villus sampling) and teratogens (thalidomide, misoprostol) have been linked to LDs, genetic factors, including chromosomal abnormalities and single gene defects are thought to be common causes [Barham and Clarke, 2008; Gold et al., 2011]. In particular, genes in a number of signaling pathways regulate different aspects of limb bud growth and patterns of development in three axes: dorsal-ventral, anterior-posterior, and proximal-distal [Barham and Clarke, 2008; Johnson and Tabin, 1997]. Disruptions in such genes or those important in vascularization or coagulation have been associated with LDs [Barham and Clarke, 2008; Carmichael et al., 2006a; Carmichael et al., 2006b; Fantel et al., 1997; Gregg et al., 1998; Hunter, 2000; Johnson and Tabin, 1997].

Based on experimental studies using vertebrate limbs, the following genes are recognized as being involved in normal human limb development: fibroblast growth factors (FGFs) *FGF4, FGF8*, and *FGF10*; sonic hedgehog (*SHH*); gremlin 1 (*GREM1*); *WNT7A*; engrailed-1 (*EN1*); LIM homeobox transcription factor 1-beta (*LMBX1*); Beta-catenin (*CTNNB1*); bone morphogenetic protein (*BMP*) genes including *GDF5*; *HOX* genes *HOX9-HOX13*; T-box (*TBX*) genes *TBX2-TBX5*; Sal-like protein 4 (*SALL4*); and cytochrome P450 genes *CYP26B1* and *CYP26A1* (through control of retinoic acid levels) [Barham and Clarke, 2008; Johnson and Tabin, 1997]. In humans, pathogenic mutations in many of these genes may cause syndromes that commonly include LDs. Examples include *WNT7A* mutations and Fuhrmann syndrome (OMIM #228930), *FGF10* mutations and lacrimo-auriculo-dento-digital syndrome (OMIM #149730), *GDF5* mutations and Du Pan syndrome (OMIM #228900), *TBX3* and ulnar-mammary syndrome (OMIM #181450), and *TBX5* and Holt-Oram syndrome (OMIM #142900).

A functioning vascular network is also essential for the progression of normal limb development. Genes regulating angiogenesis, including vascular endothelial growth factor (*VEGFA*), hypoxia-inducible factor 1 alpha (*HIF1A*) and nitric oxide synthase 3 (*NOS3*; also known as endothelial *NOS*) are important in establishing a network of blood vessels. As an example, LDs have been observed in *NOS3*-deficient mice [Gregg et al., 1998] and in rats exposed to NOS3 inhibitors [Fantel et al., 1997]. Coagulation abnormalities have been proposed as a cause of LDs. In a small study, Hunter [2000] found suggestive evidence that hypercoagulability was more common in patients with LDs.

To date, little is known about the relationship between LD and common variants in genes important for limb development, angiogenesis, and coagulation. Our objectives were to identify markers for common genetic variants that play a role in causing congenital LDs by examining single nucleotide polymorphisms (SNPs) in genes involved in limb development, angiogenesis, and coagulation. Using a population-based cohort of infants with LDs and a sample of non-malformed infants, we tested 132 SNPs in 20 candidate genes for associations with LDs.

MATERIALS AND METHODS

Study population

Case and control infants were selected from the population of live births in New York State (NYS) during the years 1998 through 2005 (N= 2,023,083). The NYS Congenital Malformations Registry (CMR) obtains reports of infants diagnosed with major congenital anomalies from hospitals statewide. The methods by which cases are ascertained by the CMR have been described previously [Sekhobo and Druschel, 2001]. CMR ascertainment of major malformations is estimated to be about 89% complete based on a comparison of CMR reports with reports from the active case-ascertainment of the National Birth Defects Prevention Study in New York State (C. Druschel unpublished data, 2002).

All infants reported to the CMR as having a LD were included in this study with the exception of those with known chromosomal anomalies or known or suspected genetic syndromes. We defined congenital LDs similarly to the definition of Gold et al. (2011) to include defects in which all or part of a “long bone, metacarpal, metatarsal, or phalanx of one or more limbs” was absent. Transverse, longitudinal, and intercalary deficiencies were defined respectively, as missing bone(s) beyond a specific point, missing bone(s) parallel to the axis of the limb, and missing bone(s) with more distal structures present.

The coding of transverse, longitudinal, and intercalary deficiencies was based on text descriptions of the malformations reported to the CMR. Inadequate detail sometimes prevented us from assigning the type of deficiency to one of these categories. If both a longitudinal deficiency and an apparent transverse deficiency were reported for the same infant, we classified the infant as having a longitudinal deficiency. In some cases, we could not distinguish a limb shortness or hypoplasia from a deficiency in which part of a limb was absent. We coded such cases as uncertain and excluded them from subanalyses.

Following an initial screening of CMR cases to exclude infants with diagnosis codes indicating the presence of a chromosomal anomaly, we identified 434 infants with LD. Review of diagnoses descriptions enabled us to make the following additional exclusions: 7 cases with chromosomal anomalies (3 trisomy 21, 1 trisomy 13, 1 trisomy 18, 1 trisomy mosaic 22, 1 Turner syndrome) and 10 with syndromes with known or suspected genetic cause with or without LDs as a component defect (2 Beckwith-Wiedemann, 1 Pena-Shokeir, 4 Holt Oram, 2 thrombocytopenia-absent radius, 1 Baller-Gerold). An additional 21 cases reported as having amniotic band “syndrome” were excluded. Following exclusions, 396 infants classified in the CMR as having LDs were included in the study.

A random sample of non-malformed control infants born 1998 to 2005 and frequency-matched 2:1 to cases on race/ethnicity was selected from NYS Newborn Screening Program records. Cases and controls were matched to NYS birth certificates to obtain data on socio-demographic factors. Archived newborn screening samples from LD case and control infants were identified and checked to determine that there was sufficient blood remaining for analysis. Specimens from three case infants could not be located or did not have sufficient blood for analysis, leaving 393 case infants available for genetic analysis. Of 1003 control infants selected, two were excluded because they were sibs of infants in the study, and bloodspots could not be retrieved for 12, leaving 989 control infants available for genetic analysis.

Personal identifying information was removed from study records before analysis. Institutional Review Board approval from the NYS Department of Health was obtained for this study. The study was reviewed by the Office of Human Subjects Research at The National Institutes of Health.

Gene selection

We selected genes that regulate various aspects of limb development and for which there was animal or human evidence that mutations caused LDs. Genes were not included if mutations only produced other types of limb abnormalities such as curvatures, contractures or extra digits. To maximize the number of genes surveyed, with two exceptions, we excluded genes that would have required more than 20 haplotype-tagging SNPs (htSNPs) to cover the gene.

A total of 14 genes involved in limb development were selected for study: SHH, WNT7A, FGF4, FGF8, FGF10, TBX3, TBX5, SALL4, GREM1, GDF5, CTNNB1, EN1, CYP26A1, and *CYP26B1*. Three genes judged to be important regulators of angiogenesis (*VEGFA, HIF1A*, and *NOS3*) and three genes for which diagnostic testing of coagulation abnormalities is clinically available (*F2, F5*, and *MTHFR*) were also selected.

SNP selection

For each gene, the UCSC Genome Browser; assembly: Mar 2006 (NCBI36/ hg18);http://genome.ucsc.edu/; was used to visualize the gene and identify the region 5kB upstream and 2kB downstream of the gene. htSNPs were selected within the defined gene region. Genotype data for SNPs in the gene region, based on the HapMap CEU population of northern and western European ancestry, were downloaded from HapMap Data Release 27 – Phase II + III, Feb09, on NCBI B36. The Tagger program was used via Haploview version 4.2 to select htSNPs with a minor allele frequency of ≥0.05 and r^2<0.8 [Barrett, 2009]. For genes involved in coagulation abnormalities, rather than htSNPs, we genotyped established functional variants associated with thrombophilia [Kupferminc et al., 1999]: *MTHFR* 677C>T (rs1801133), the Factor V Leiden mutation (*F5* rs6025), *F5* rs1800595 (4070A>G) representing the *F5* HR2 haplotype, and prothrombin 20210 G>A (*F2* rs1799963). We used the SNPnexus database [Chelala et al., 2009] to determine the possible functional consequences of SNPs that were associated with LDs in our analysis.

Laboratory Methods

Genomic DNA was extracted from 3 mm dried blood spot punches using a laboratory-developed protocol for DNA extraction using sodium hydroxide precipitation described previously [Mills et al., 2012]. At least 30 ng of the extracted DNA was whole-genome amplified by KBiosciences (Herts, UK) using a primer extension pre-amplification (PEP) method. To maximize the genotype call rate and to minimize potential errors introduced by the whole genome amplification, for each subject, two amplifications were carried out and the amplification products were each genotyped separately. SNPs were genotyped by KBiosciences using KASPar technology (proprietary fluorescent-based competitive allelic discrimination assays). Genotyping was initially attempted on 140 SNPs; results were obtained on 126. Following selection of replacement htSNPs and redesign of some assays, results for an additional six SNPs passed quality control checks. Overall, eight SNP assays did not pass quality control measures of the genotyping facility and were dropped from the study. High quality genotypes were obtained for 132 SNPs, in which call rates were >97%, and no discordant genotypes were detected when comparing results from the two independent amplification reactions or when repeat genotyping was performed on >4% of the samples. Four cases and nine controls with low call rates (<20%) for all SNPs were excluded from analysis, leaving a final study population of 389 cases and 980 controls.

Hardy-Weinberg equilibrium was tested among controls separately by race/ethnic group for all 132 SNPs (p<0.000095 was considered significant based on correction for 528 tests). *WNT7A* rs11128663 deviated significantly from Hardy-Weinberg expectations among non-Hispanic black controls and was removed from analyses for all race/ethnicities combined

and those restricted to non-Hispanic black infants. None of the other SNPs deviated from Hardy-Weinberg equilibrium in any of the race/ethnic groups.

Statistical analysis

Genotype distributions overall and by race/ethnic group were calculated; the minor allele frequency (MAF) was based on the controls overall. The main analysis included all race/ethnicities (non-Hispanic white, non-Hispanic black, Hispanic, Asian, other) and all LD phenotypes. Race/ethnic group-specific and phenotype-specific analyses were also conducted. We used unconditional logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). In analyses of all race/ethnic groups combined, adjusted ORs were calculated with race/ethnicity included as the only covariate in the logistic regression models. An unrestricted genetic model was employed in which two ORs were calculated, one comparing the heterozygous genotype to the homozygous major allele genotype (reference) and the second comparing the homozygous minor allele genotype to the homozygous major allele genotype. We corrected for 132 tests using the Bonferroni method to adjust for multiple testing. Analyses were performed using SAS software, version 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

Of the 389 infants with LDs on whom genotype data were available, isolated LDs were reported for 301; the remaining 88 had multiple congenital anomalies (one or more additional major anomalies of other organ systems). The type of LD was classified as transverse (164; 42%), longitudinal (159; 41%), or intercalary (43; 11%). Twenty-three (6%) were “unknown” because the type of deficiency could not be determined based on the CMR description provided.

Maternal and infant characteristics were fairly similar between case and control infants. There were lower proportions of multiple births and male infant sex among control infants (Table I). Overall, 57% of case infant mothers were non-Hispanic white, 18% were non-Hispanic black, 19% were Hispanic, 4% were Asian, and 2% were “other” race/ethnicity. The race/ethnicity distributions by LD phenotype are shown in Table II.

The genotype distributions overall and by race/ethnic group for each of the 132 SNPs assayed are presented in Supplemental eTable I (see Supporting Information online). Genotype frequencies often varied by race/ethnicity. For some SNPs, the major and minor alleles were reversed for certain race/ethnic groups; however, the major allele among all control infants was used as the reference allele for all analyses.

Logistic regression results overall and for race/ethnic- and phenotype-specific analyses for each of the 132 SNPs tested are available in Supplemental eTables II-IV (see Supporting Information online). In Table III, the results for all race/ethnic groups and all phenotypes combined are presented for SNPs for which a nominally significant result ($p < 0.05$) was observed for one of the genotype comparisons. Adjusted for race/ethnicity, nominally significant results were observed for *EN1, FGF10, SHH, TBX5, VEGFA*, and *NOS3* SNPs. The strongest findings were for *FGF10* rs10805683, *FGF10* rs13170645 and *EN1* rs893574. Adjusted ORs for the heterozygous and homozygous minor allele genotypes were 1.49 (95% CI: 1.16-1.92; p=0.0017) and 1.88 (95% CI: 1.13-3.12; p=0.015) for *FGF10* rs10805683 and 1.47 (95% CI: 1.10-1.95; p=0.0089) and 1.83 (95% CI: 1.30-2.59; p=0.0006) for *FGF10* rs13170645. For *EN*1 rs893574, the adjusted OR (aOR) for the heterozygous genotype was 1.66 (95% CI: 1.16-2.38; p=0.0059) and the aOR for the homozygous minor allele genotype was more elevated but nonsignificant due to the small number of homozygous individuals (MAF = 5.0% among controls). Among non-Hispanic white infants, the associations with

FGF10 SNPs remained significant after correction for multiple testing (Table IV). The estimates are as follows: 1.99 (95% CI: 1.43-2.77; uncorrected p=0.000043, corrected p=0.0057) for the *FGF10* rs10805683 heterozygous genotype, and 2.37 (95% CI: 1.48-3.78; uncorrected p=0.00032, corrected p=0.0422) for the *FGF10* rs13170645 homozygous minor genotype. For both SNPs, estimates for two copies of the minor allele were farther from the null than those for only one copy. In the four major racial/ethnic groups studied, linkage disequilibrium between the two *FGF10* SNPs was minimal to modest (D'=0.70-1.0, r^2=0.07-0.43). In the non-Hispanic white population, where the association of the *FGF10* SNPs with LDs was the strongest, linkage disequilibrium was high (D'=0.98, r^2=0.43).

Table IV presents results for SNPs for which a nominally significant result was observed for at least one genotype comparison for any race/ethnic group. Among non-Hispanic white infants, significant ORs were noted for *CYP26B1, WNT7A*, and *FGF8* SNPs in addition to genes associated with limb deficiencies in the main analysis (*EN1, FGF10, SHH*, and *TBX5*).

Among non-Hispanic black infants, there were a number of significantly reduced ORs for SNPs in genes *CYP26B1, EN1, WNT7A, TBX5, TBX3*, and *SALL4*, along with one significantly elevated OR for a *WNT7A* SNP. Significantly increased ORs were observed for *EN1, WNT7A, TBX5, GDF5, SALL4, VEGFA*, and *NOS3*, along with a significantly reduced OR for two *WNT7A* SNPs among Hispanic infants. Among Asian infants, several significant associations with relatively high ORs (range: 5.6-39.6) were noted for one SNP each in *EN1, WNT7A*, and *TBX5*, and two *HIF1A* SNPs.

Table V presents results for SNPs for which a nominally significant result was observed for the heterozygous and/or homozygous phenotype for at least one of the three LD phenotypes examined. The aOR between SNPs and transverse limb deficiencies was significantly reduced for one *EN1* SNP and three *WNT7A* SNPs and increased for one *EN1, TBX5*, and *TBX3* SNP, four *WNT7A* SNPs and three *FGF10* SNPs. Analysis of longitudinal LDs showed significantly increased aORs in *FGF10* (two SNPs), *SHH, SALL4, VEGFA, NOS3*, and *HIF1A* as well as significantly reduced aORs in *GREM1* and *NOS3*. Significant positive associations were observed between *FGF10, FGF4*, and *TBX5* (two SNPs) and intercalary LDs, and significant negative associations were observed for *TBX5* and *NOS3*. The two *FGF10* SNPs that were significantly associated with all LDs combined among non-Hispanic white infants were nominally significant for the heterozygous and/or homozygous variant genotype for both transverse and longitudinal LDs (all race/ethnicities combined). None of the estimates from analyses restricted to specific LD phenotypes remained significant after correction for multiple testing.

We observed that *WNT7A* rs3762721, rs9863149, and rs1946620 were associated with transverse deficiencies and with all LDs among Hispanic infants. Therefore, we performed an analysis of transverse deficiencies among Hispanic infants. We found that ORs for the heterozygous and homozygous minor genotypes of *WNT7A* rs3762721, rs9863149, and rs1946620 were 2.30 (95% CI: 0.83-6.33; p=0.1074), 5.27 (95% CI: 1.66-16.77; p=0.0049); 3.89 (95% CI: 1.38-11.01; p=0.0104), 7.92 (95% CI: 2.00-31.39; p=0.0032), and 2.47 (95% CI: 0.75-8.11; p=0.1358), 5.46 (95% CI: 1.65-18.07; p=0.0055); respectively.

No associations were observed between variant genotypes and LDs for *CTNNB1* or *CYP26A1* SNPs or any of the "coagulation SNPs" (*MTHFR, F5*, or *F2*) in the main analysis or in race/ethnicity- or phenotype-specific analyses. An analysis excluding 39 cases for which it was somewhat uncertain whether part of a limb was missing or just small in size produced results similar to those for the main analysis (data not shown).

DISCUSSION

Our strongest findings were for the *FGF10* rs10805683 and *FGF10* rs13170645 SNPs among non-Hispanic white infants. Associations for these SNPs were statistically significant following a very conservative correction for multiple testing. Moreover, nominally significant findings were observed in all three LD phenotypes (transverse, longitudinal, intercalary) for *FGF10* rs10805683 and for transverse and longitudinal deficiencies for *FGF10* rs13170645 (ORs were elevated but not significant for intercalary deficiencies). Modest linkage disequilibrium was observed between the two significant *FGF10* SNPs and both are intronic. These htSNPs (located in intron 1) may be markers for one or more causal SNPs or they may be in a regulatory region of the *FGF10* gene. We searched for variants in nearby regulatory regions of *FGF10* because such variants could have functional effects and may be in linkage disequilibrium with the htSNPs. None of 10 known SNPs within a 1097-bp intron 1 enhancer region of *FGF10* [Golzio et al., 2012] were genotyped in HapMap, but a SNP 334-bp downstream of the enhancer (rs1482679) is in perfect linkage disequilibrium with the most significant *FGF10* SNP, rs13170645 (HapMap CEU population). Both mouse and human data support our findings and suggest that *FGF10* variants play a causal role in limb defects. In *fgf10* null mutant mice, development of limbs, lungs and a number of other organs is severely affected [Ohuchi et al., 2000; Sekine et al., 1999]. In humans, *FGF10* mutations have been found in patients with lacrimo-auriculo-dento-digital (LADD) syndrome [Milunsky et al., 2006].

For the genes involved in regulating limb development, we observed nominally significant associations between selected *EN1, FGF10, SHH*, and *TBX5* SNPs and LDs in the analysis of all race/ethnicities and all phenotypes combined. Associations emerged for additional genes in race/ethnic group-specific analyses (*CYP26B1, WNT7A, FGF8, TBX3, GDF5, SALL4*) and in analyses by LD phenotype (*WNT7A, FGF4, TBX3, GREM1, SALL4*). The importance of each of these genes in limb development has been demonstrated in experimental studies using vertebrate limb models [Barham and Clarke, 2008; Johnson and Tabin, 1997].

Most of the variants tested in this study are common haplotype-tagging SNPs found in non-coding regions of the genome; however, several in coding regions or that are predicted to have functional relevance were nominally significant [Chelala et al., 2009]. For example, *CYP26B1* rs2241057 codes for an amino acid change (p.L264S), and *WNT7A* rs12639607 results in a synonymous change (p.A105A). SNP rs3796316, downstream of the *WNT7A* gene, and rs551510, in a *TBX3* intron, are both in predicted transcription factor binding sites. Three SNPs are in or near CpG islands (rs9309462 in a *CYP26B1* intron, *WNT7A* rs12639607, and rs6709773 upstream of *EN1*), and thus may be involved in regulation of transcription. Several variants, including two that were significant in the overall analysis (*EN1* rs3754855 and *VEGFA* rs3025040) are in conserved regions. SNP rs3825214 in *TBX5* was found to be associated with electrocardiographic traits in one GWAS [Holm et al., 2010]. Functional consequences of the other SNPs for which at least nominally significant associations were observed remain to be determined.

A pilot study by Hunter [2000] measured various biochemical indicators of thrombophilia as well as diagnostic mutations in *MTHFR, F5*, and *F2* among children with transverse LDs and their mothers. A greater than expected number of children were heterozygous for the *MTHFR* variant but no differences were observed for the homozygous *MTHFR* genotype or variants in *F5* or *F2* compared with the prevalences in the general population; however, only 24 children were included in the study. Another study, by Carmichael et al. [2006a] examined three SNPs also tested in our study: one SNP each for *MTHFR, F5*, and *F2*. Carmichael et al. observed an elevated OR for the *F5* rs6025 heterozygous genotype

whereas in our study the OR was not significantly increased. Similar to our study, Carmichael et al. [2006a] observed null results for the *MTHFR* SNP.

We observed a few associations in genes involved in angiogenesis (*VEGFA, NOS3*, and *HIF1A*), none of which remained significant following correction for the total number of SNPs tested in our study. High ORs were observed for the homozygous minor allele genotypes of two *HIF1A* SNPs among Asians with ORs of 7.50 (95% CI: 1.53-36.71; p=0.0129) and 39.59 (95% CI: 3.90-401.49; p=0.00185). However, these estimates were based on small numbers (17 cases and 46 controls). Despite an expectation that reduced function of angiogenesis genes might contribute to transverse LDs in particular, via a hypoxia mechanism, no associations were observed in an analysis restricted to transverse LDs (all race/ethnicities combined).

There is good biological evidence to support our finding that *CYP26B1* and *WNT7A* are important in the causation of LDs. Retinoic acid levels affect various aspects of embryonic development including limb development and the *CYP26B1* enzyme degrades retinoic acid [Pennimpede et al., 2010]. Further research on the implications of the amino acid change caused by the *CYP26B1* rs2241057 variant allele on enzyme function would be useful in evaluating a causal role for this variant in LDs. For the *WNT7A* gene, seven nominally significant findings were observed for transverse LDs. Based on findings of causal *WNT7A* mutations in syndromes with LDs of varying severity, along with gene expression study results, Woods et al. [2006] suggested that in humans the *WNT7A* gene is important in maintaining both the zone of polarizing activity (ZPA) and apical ectodermal ridge (AER) [Woods et al., 2006]. Because we observed associations among Hispanic infants (all phenotypes) and several of these SNPs, we conducted a subanalysis of *WNT7A* variants and transverse limb deficiencies among Hispanic infants and found estimates farther from the null for each of the three SNPs that were nominally significant in race/ethnic- and phenotype-specific analyses. This subanalysis is based on small numbers but provides support for *WNT7A* involvement in transverse limb deficiencies. Various *WNT7A* SNPs were nominally significant in all four race/ethnic groups, providing further suggestive evidence for this gene.

We are aware of only one previous epidemiologic study of genes involved in angiogenesis and limb deficiencies. Carmichael et al. [2006b] reported elevated ORs for the heterozygous and homozygous *NOS3* rs1800783 genotypes and for the heterozygous *NOS3* rs1799983 genotype. Despite a much larger sample size (four times as many cases and controls), we did not detect associations for either of these SNPs.

We used a Bonferroni correction for the 132 SNPs included in this study. We think this is a fairly conservative correction given that each of the 14 developmental genes (104 htSNPs) is known to regulate limb development and the remaining 6 (28 SNPs) play a role in angiogenesis or coagulation. Given that we tested *a priori* hypotheses based on literature pointing to the importance of these genes, even significant findings that did not survive multiple testing correction provide suggestive evidence for genes and SNPs deserving further study.

Strengths of our study are the large study population, the population-based design (ascertainment of infants with non-syndromic LDs statewide and random selection of unaffected infants from the same base population), and high-quality genotyping data. Limitations of our study should also be noted. Despite the large number of cases overall, there were relatively small numbers of subjects in some subgroups in analyses stratified by race/ethnicity and phenotype. Population stratification is also likely to be an issue, especially in the Hispanic sample. In addition, we selected htSNPs based on the allele distributions in

the HapMap European population. Resources prevented selecting additional htSNPs to adequately cover each gene for each race/ethnic group. Another limitation was that case ascertainment was based on narrative descriptions of birth defects as reported by hospital medical records personnel to the New York Congenital Malformations Registry. Classifying cases by the type of LD was inexact if insufficient detail was reported.

CONCLUSION

Animal studies have shown that *FGF10* induces formation of the apical ectodermal ridge and is necessary for limb development. Our data suggest that common variants in *FGF10* increase the risk for a wide range of non-syndromic LDs. Future studies should focus on identification of functional variants in linkage disequilibrium with the two *FGF10* SNPs that are responsible for the associations detected in this study; next steps would include functional studies in relevant tissues. Our findings also provide suggestive evidence for SNPs in other genes including *CYP26B1* and *WNT7A*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

Barham G, Clarke NM. Genetic regulation of embryological limb development with relation to congenital limb deformity in humans. J Child Orthop. 2008; 2:1–9. [PubMed: 19308596]

Barrett JC. Haploview: Visualization and analysis of SNP genotype data. Cold Spring Harb Protoc. 2009 2009:db.

Carmichael SL, Shaw GM, Iovannisci DM, Yang W, Finnell RH, Cheng S, Lammer EJ. Risks of human limb deficiency anomalies associated with 29 SNPs of genes involved in homocysteine metabolism, coagulation, cell-cell interactions, inflammatory response, and blood pressure regulation. Am J Med Genet A. 2006a; 140:2433–2440. [PubMed: 17036337]

Carmichael SL, Shaw GM, Yang W, Iovannisci DM, Lammer E. Risk of limb deficiency defects associated with *NAT1, NAT2, GSTT1, GSTM1*, and *NOS3* genetic variants, maternal smoking, and vitamin supplement intake. Am J Med Genet A. 2006b; 140:1915–1922. [PubMed: 16906563]

Chelala C, Khan A, Lemoine NR. SNPnexus: a web database for functional annotation of newly discovered and public domain single nucleotide polymorphisms. Bioinformatics. 2009; 25:655–661. [PubMed: 19098027]

Fantel AG, Nekahi N, Shepard TH, Cornel LM, Unis AS, Lemire RJ. The teratogenicity of N(omega)-nitro-L-ariginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, in rats. Reprod Toxicol. 1997; 11:709–717. [PubMed: 9311580]

Gold NB, Westgate MN, Holmes LB. Anatomic and etiological classification of congenital limb deficiencies. Am J Med Genet A. 2011; 155A:1225–1235. [PubMed: 21557466]

Golzio C, Havis E, Daubas P, Nuel G, Babarit C, Munnich A, Vekemans M, Zaffran S, Lyonnet S, Etchevers HC. ISL1 directly regulates *FGF10* transcription during human cardiac outflow formation. PLoS One. 2012; 7:e30677. [PubMed: 22303449]

Gregg AR, Schauer A, Shi O, Liu Z, Lee CG, O'Brien WE. Limb reduction defects in endothelial nitric oxide synthase-deficient mice. Am J Physiol. 1998; 275:H2319–H2324. [PubMed: 9843834]

Holm H, Gudbjartsson DF, Arnar DO, Thorleifsson G, Thorgeirsson G, Stefansdottir H, Gudjonsson SA, Jonasdottir A, Mathiesen EB, Njolstad I, Nyrnes A, Wilsgaard T, Hald EM, Hveem K, Stoltenberg C, Lochen ML, Kong A, Thorsteinsdottir U, Stefansson K. Several common variants modulate heart rate, PR interval and QRS duration. Nat.Genet. 2010; 42:117–122. [PubMed: 20062063]

Hunter AG. A pilot study of the possible role of familial defects in anticoagulation as a cause for terminal limb reduction malformations. Clin Genet. 2000; 57:197–204. [PubMed: 10782926]

Johnson RL, Tabin CJ. Molecular models for vertebrate limb development. Cell. 1997; 90:979–990. [PubMed: 9323126]

Kupferminc MJ, Eldor A, Steinman N, Many A, Bar-Am A, Jaffa A, Fait G, Lessing JB. Increased frequency of genetic thrombophilia in women with complications of pregnancy. N Engl J Med. 1999; 340:9–13. [PubMed: 9878639]

Mills JL, Carter TC, Kay DM, Browne ML, Brody LC, Liu A, Romitti PA, Caggana M, Druschel CM. Folate and vitamin B12-related genes and risk for omphalocele. Hum.Genet. 2012; 131:739–746. [PubMed: 22116453]

Milunsky JM, Zhao G, Maher TA, Colby R, Everman DB. LADD syndrome is caused by *FGF10* mutations. Clin Genet. 2006; 69:349–354. [PubMed: 16630169]

Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, Itoh N. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. Biochem Biophys.Res Commun. 2000; 277:643–649. [PubMed: 11062007]

Pennimpede T, Cameron DA, MacLean GA, Li H, bu-Abed S, Petkovich M. The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis. Birth Defects Res A Clin Mol Teratol. 2010; 88:883–894. [PubMed: 20842651]

Sekhobo JP, Druschel CM. An evaluation of congenital malformations surveillance in New York State: an application of Centers for Disease Control and Prevention (CDC) guidelines for evaluating surveillance systems. Public Health Rep. 2001; 116:296–305. [PubMed: 12037258]

Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. *Fgf10* is essential for limb and lung formation. Nat.Genet. 1999; 21:138–141. [PubMed: 9916808]

Stoll C, Alembik Y, Dott B, Roth MP. Associated malformations in patients with limb reduction deficiencies. Eur J Med Genet. 2010; 53:286–290. [PubMed: 20670696]

Woods CG, Stricker S, Seemann P, Stern R, Cox J, Sherridan E, Roberts E, Springell K, Scott S, Karbani G, Sharif SM, Toomes C, Bond J, Kumar D, Al-Gazali L, Mundlos S. Mutations in *WNT7A* cause a range of limb malformations, including Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome. Am J Hum.Genet. 2006; 79:402–408. [PubMed: 16826533]

Table I
Selected Characteristics of Infants with Limb Deficiencies and Control Infants

	Cases N=389		Controls N=980	
	N*	%	N*	%
Maternal age (years)				
<20	39	10.0	78	8.0
20-24	73	18.8	176	18.0
25-29	97	24.9	252	25.7
30-39	164	42.2	434	44.3
40+	16	4.1	40	4.1
Maternal race/ethnicity				
White non-Hispanic	221	56.8	543	55.4
Black non-Hispanic	69	17.7	180	18.4
Hispanic	75	19.3	191	19.5
Asian	17	4.4	46	4.7
Other	7	1.8	20	2.0
Maternal education (years)				
<12	80	20.9	160	16.5
12	120	31.4	285	29.4
13-15 16	82 54	21.5 14.1	216 166	22.2 17.1
17+	46	12.0	144	14.8
Maternal prepregnancy BMI†				
<18.5	29	12.0	45	8.1
18.5-<25	129	53.3	298	53.6
25-<30	34	14.0	116	20.9
30+	50	20.7	97	17.4
Plurality‡				
Singleton	368	94.6	956	97.6
Twin	20	5.1	23	2.4
Triplet	1	0.3	1	0.1
Infant sex‡				
Male	219	56.3	487	49.7
Female	170	43.7	493	50.3
Limb deficiency phenotype				
Transverse	164	42.2		
Longitudinal	159	40.9		
Intercalary	43	11.1		
Unknown§	23	5.9		

Abbreviation: BMI=body mass index (weight in kg/height in m^2).

[*] Total counts vary due to missing values for some variables

[†] Data are missing for births occurring in New York City (147 cases and 424 controls).

[‡] Chi-square p-value <0.05.

[§] Could not be definitively classified by type of deficiency.

Table II

Race/Ethnicity Distribution by Limb Deficiency Phenotype

	Transverse (n=164)		*Longitudinal (n=159)*		*Intercalary (n=43)*		*Unknown (n=23)*	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Non-Hispanic white	105	64.0	88	55.3	22	51.2	6	26.1
Non-Hispanic black	25	15.2	30	18.9	9	20.9	5	21.7
Hispanic	27	16.5	27	17.0	11	25.6	10	43.5
Asian	6	3.7	10	6.3	1	2.3	0	0
Other	1	0.6	4	2.5	0	0	2	8.7

Table III

Associations between SNPs and Congenital Limb Deficiencies*

		Heterozygous		Homozygous minor	
gene	**SNP**	**OR (CI)***	**p-value**	**OR (CI)***	**p-value**
Development					
EN1	rs3754855	**0.73 (0.57-0.95)**	**0.01930**	0.86 (0.60-1.22)	0.38363
EN1	rs893574	**1.66 (1.16-2.38)**	**0.00590**	2.88 (0.40-20.66)	0.29383
FGF10	rs10805683	**1.49 (1.16-1.92)**	**0.00167**	**1.88 (1.13-3.12)**	**0.01508**
FGF10	rs13170645	**1.47 (1.10-1.95)**	**0.00890**	**1.83 (1.30-2.59)**	**0.00060**
SHH	rs208684	0.95 (0.74-1.22)	0.70254	**1.83 (1.13-2.96)**	**0.01433**
SHH	rs1233556	1.15 (0.88-1.49)	0.30684	**0.37 (0.14-0.94)**	**0.03770**
TBX5	rs2295234	**1.37 (1.05-1.79)**	**0.02121**	1.10 (0.50-2.43)	0.81193
Angiogenesis					
VEGFA	rs3025040	1.01 (0.77-1.32)	0.96448	**0.41 (0.17-0.99)**	**0.04751**
NOS3	rs1800781	**0.69 (0.51-0.95)**	**0.02368**	1.53 (0.62-3.79)	0.35964

Abbreviations: SNP=single nucleotide polymorphism, OR=odds ratio, CI=95% confidence interval.

*All race/ethnicity groups and all types of limb deficiencies combined (389 infants with limb deficiencies and 980 unaffected infants). Results are presented for SNPs for which a nominally significant result ($p < 0.05$) was observed for one of the genotype comparisons.

†Odds ratios are adjusted for race/ethnicity; the reference group is the homozygous major genotype.

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Table IVa

Associations between SNPs and Limb Deficiencies by Race/Ethnic Group*

		NHW				NHB			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	SNP	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)*	p-value
Development									
CYP26B1	rs9309462	1.43 (0.89-2.30)	0.14214	**10.47 (1.16-94.30)**	**0.03621**	0.82 (0.44-1.55)	0.54885	0.69 (0.14-3.46)	0.65574
CYP26B1	rs2241057	1.40 (0.98-1.99)	0.06581	**3.75 (1.55-9.09)**	**0.00342**	0.77 (0.43-1.38)	0.38455	0.56 (0.18-1.80)	0.33321
CYP26B1	rs11898950	1.18 (0.85-1.64)	0.33247	1.70 (0.84-3.43)	0.13757	0.72 (0.41-1.28)	0.26194	**0.13 (0.02-0.99)**	**0.04915**
EN1	rs6709773	0.93 (0.66-1.30)	0.66395	1.21 (0.73-2.03)	0.46138	1.77 (0.96-3.28)	0.06936	1.85 (0.80-4.25)	0.14822
EN1	rs893574	**1.97 (1.14-3.39)**	**0.01498**	NE		1.15 (0.57-2.32)	0.68622	NE	
EN1	rs13023152	0.95 (0.67-1.34)	0.77202	1.51 (0.85-2.69)	0.16262	**0.42 (0.22-0.81)**	**0.00967**	2.08 (0.64-6.80)	0.22388
WNT7A	rs9819887	1.06 (0.76-1.48)	0.73448	1.21 (0.69-2.12)	0.51117	**0.40 (0.17-0.95)**	**0.03686**	0.78 (0.34-1.80)	0.55912
WNT7A	rs12639607	0.79 (0.55-1.14)	0.21093	**2.67 (1.01-7.06)**	**0.04689**	1.28 (0.57-2.87)	0.55646	NE	
WNT7A	rs3762720	1.15 (0.81-1.64)	0.42779	1.07 (0.68-1.67)	0.77872	0.64 (0.31-1.34)	0.24177	NE	
WNT7A	rs3762721	1.33 (0.93-1.90)	0.12022	0.61 (0.17-2.16)	0.44199	0.70 (0.32-1.55)	0.37785	0.98 (0.43-2.26)	0.97129
WNT7A	rs12492784	0.82 (0.59-1.15)	0.25873	0.74 (0.46-1.21)	0.23405	0.94 (0.48-1.81)	0.84300	1.03 (0.47-2.26)	0.93815
WNT7A	rs9863149	1.16 (0.83-1.61)	0.37758	1.19 (0.68-2.09)	0.54157	1.11 (0.58-2.14)	0.74717	1.06 (0.50-2.27)	0.87444
WNT7A	rs17038695	**0.65 (0.44-0.96)**	**0.02952**	NE		0.77 (0.42-1.38)	0.37685	2.46 (0.68-8.96)	0.17159
WNT7A	rs12634112	1.23 (0.85-1.78)	0.26725	0.70 (0.19-2.55)	0.59001	0.94 (0.52-1.69)	0.83149	0.99 (0.38-2.59)	0.98349
WNT7A	rs6803033	0.75 (0.51-1.10)	0.13675	NE		0.88 (0.48-1.62)	0.68256	**3.35 (1.33-8.47)**	**0.01045**
WNT7A	rs9840696	1.04 (0.74-1.47)	0.80312	1.05 (0.65-1.68)	0.85100	0.87 (0.44-1.72)	0.68071	1.27 (0.11-14.28)	0.84728
WNT7A	rs1946620	1.21 (0.86-1.70)	0.27141	1.17 (0.72-1.91)	0.52252	0.89 (0.39-2.04)	0.78982	0.70 (0.31-1.56)	0.38050
FGF10	rs10805683	**1.99 (1.43-2.77)**	**0.00004**	**2.52 (1.08-5.89)**	**0.03341**	0.85 (0.46-1.57)	0.61454	1.26 (0.44-3.58)	0.66199
FGF10	rs13170645	1.36 (0.96-1.93)	0.08039	**2.37 (1.48-3.78)**	**0.00032**	1.72 (0.63-4.65)	0.28820	1.46 (0.54-3.91)	0.45227
SHH	rs208684	0.86 (0.62-1.20)	0.38166	**1.96 (1.06-3.62)**	**0.03187**	1.08 (0.60-1.96)	0.79597	1.74 (0.67-4.55)	0.25828
FGF8	rs35344824	**1.62 (1.03-2.55)**	**0.03706**	1.76 (0.29-10.62)	0.53760	NE		NE	
TBX5	rs2295234	**1.87 (1.32-2.64)**	**0.00041**	0.90 (0.29-2.80)	0.85431	0.60 (0.26-1.39)	0.23470	1.21 (0.11-13.58)	0.87815
TBX5	rs1996821	1.33 (0.96-1.85)	0.08909	1.74 (0.82-3.69)	0.14759	0.63 (0.34-1.16)	0.13547	**0.36 (0.15-0.88)**	**0.02425**
TBX5	rs2236017	0.92 (0.65-1.30)	0.64171	0.85 (0.53-1.38)	0.52169	0.67 (0.33-1.38)	0.28216	0.75 (0.34-1.65)	0.46759
TBX5	rs7964303	0.92 (0.66-1.28)	0.61459	1.02 (0.59-1.77)	0.93080	1.11 (0.62-1.98)	0.72171	0.49 (0.16-1.55)	0.22485

		NHW				NHB			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	**SNP**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)***	**p-value**
TBX5	rs1946293	0.89 (0.64-1.25)	0.50066	1.04 (0.60-1.81)	0.89505	0.56 (0.30-1.03)	0.06052	**0.36 (0.15-0.88)**	**0.02456**
TBX5	rs3825214	0.94 (0.66-1.34)	0.72531	0.74 (0.36-1.55)	0.42833	**0.51 (0.28-0.94)**	**0.02991**	0.45 (0.12-1.65)	0.22579
TBX3	rs551510	1.04 (0.71-1.54)	0.82632	1.34 (0.86-2.08)	0.20205	**0.40 (0.20-0.81)**	**0.01075**	0.76 (0.38-1.55)	0.45332
GDF5	rs224333	0.94 (0.67-1.32)	0.71434	1.06 (0.66-1.71)	0.80529	0.93 (0.16-5.51)	0.93645	0.71 (0.13-3.99)	0.69409
SALL4	rs2179597	1.04 (0.72-1.50)	0.82322	0.86 (0.54-1.37)	0.51546	**0.37 (0.14-0.95)**	**0.03852**	0.55 (0.22-1.37)	0.20138
SALL4	rs6021451	0.86 (0.61-1.22)	0.39145	0.69 (0.25-1.91)	0.47427	**0.37 (0.19-0.71)**	**0.00268**	1.45 (0.48-4.43)	0.51098
SALL4	rs6021435	1.05 (0.76-1.46)	0.76235	0.90 (0.50-1.64)	0.73677	0.69 (0.33-1.45)	0.33061	1.42 (0.66-3.04)	0.37111
Angiogenesis									
VEGFA	rs3025053	0.85 (0.55-1.31)	0.46678	1.60 (0.27-9.66)	0.60814	0.46 (0.10-2.12)	0.31893	NE	
NOS3	rs2853796	0.92 (0.63-1.32)	0.64076	0.87 (0.55-1.39)	0.57113	0.76 (0.37-1.57)	0.45606	1.23 (0.56-2.71)	0.61117
HIF1A	rs12434438	0.88 (0.63-1.23)	0.46514	0.81 (0.43-1.54)	0.52141	1.76 (0.35-8.79)	0.49266	1.41 (0.29-6.94)	0.67341
HIF1A	rs2301113	0.88 (0.63-1.22)	0.43233	1.08 (0.59-1.96)	0.80200	1.01 (0.18-5.62)	0.99128	0.94 (0.18-5.03)	0.94486

Abbreviations: SNP=single nucleotide polymorphism, NHW=non-Hispanic white, NHB=non-Hispanic black, OR=odds ratio, CI=95% confidence interval, NE=not estimable: no cases and/or no control infants with this genotype.

*Results are presented for SNPs for which a nominally significant result ($p < 0.05$) was observed for at least one genotype comparison for any race/ethnic group.

†The reference group is the homozygous major genotype

Table IVb

Associations between Gene Variants and Limb Deficiencies by Race/Ethnic Group *

		Hispanic				Asian			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	SNP	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value
Development									
CYP26B1	rs9309462	1.92 (0.87-4.26)	0.10630	NE		NE		NE	
CYP26B1	rs2241057	1.16 (0.67-2.01)	0.59094	0.32 (0.07-1.46)	0.14131	1.71 (0.36-8.12)	0.49682	NE	
CYP26B1	rs11898950	1.09 (0.62-1.90)	0.76449	0.54 (0.17-1.68)	0.28633	3.85 (0.93-16.01)	0.06355	0.44 (0.04-4.82)	0.50492
EN1	rs6709773	1.36 (0.74-2.50)	0.31991	1.18 (0.54-2.57)	0.67057	2.59 (0.69-9.70)	0.15853	**7.67 (1.22-48.04)**	**0.02961**
EN1	rs893574	**2.39 (1.02-5.61)**	**0.04516**	NE		1.25 (0.36-4.35)	0.72571	NE	
EN1	rs13023152	1.04 (0.59-1.82)	0.89063	0.91 (0.33-2.48)	0.84771	0.79 (0.23-2.70)	0.70658	0.36 (0.04-3.39)	0.36979
WNT7A	rs9819887	0.89 (0.44-1.81)	0.74555	1.89 (0.89-4.01)	0.09706	0.22 (0.04-1.22)	0.08284	0.24 (0.04-1.37)	0.10789
WNT7A	rs12639607	0.74 (0.40-1.37)	0.33203	0.51 (0.11-2.43)	0.39770	0.43 (0.12-1.53)	0.19341	1.20 (0.23-6.19)	0.82749
WNT7A	rs3762720	**0.49 (0.27-0.87)**	**0.01439**	0.28 (0.08-1.00)	0.05069	2.20 (0.63-7.69)	0.21711	3.67 (0.21-64.55)	0.37461
WNT7A	rs3762721	1.50 (0.82-2.75)	0.18560	**2.64 (1.20-5.79)**	**0.01573**	0.45 (0.12-1.67)	0.23149	0.64 (0.11-3.69)	0.61447
WNT7A	rs12492784	**1.85 (1.05-3.26)**	**0.03456**	1.28 (0.49-3.34)	0.61975	0.93 (0.30-2.87)	0.90115	NE	
WNT7A	rs9863149	1.51 (0.85-2.68)	0.15671	**2.83 (1.11-7.23)**	**0.03004**	0.83 (0.24-2.90)	0.76585	2.27 (0.45-11.35)	0.31942
WNT7A	rs17038695	1.25 (0.63-2.48)	0.51823	1.78 (0.29-10.94)	0.53255	2.67 (0.62-11.53)	0.18930	NE	
WNT7A	rs12634112	1.19 (0.67-2.13)	0.54608	**7.17 (1.34-38.24)**	**0.02116**	0.54 (0.10-2.82)	0.46480	NE	
WNT7A	rs6803033	1.40 (0.75-2.58)	0.28826	1.79 (0.56-5.72)	0.32864	**7.33 (1.99-26.96)**	**0.00271**	4.40 (0.58-33.21)	0.15085
WNT7A	rs9840696	0.65 (0.37-1.14)	0.13432	**0.09 (0.01-0.71)**	**0.02180**	1.25 (0.38-4.04)	0.71328	NE	
WNT7A	rs1946620	1.61 (0.84-3.07)	0.14969	**2.11 (1.02-4.36)**	**0.04456**	0.45 (0.11-1.89)	0.27825	1.67 (0.41-6.77)	0.47491
FGF10	rs10805683	1.23 (0.69-2.17)	0.48240	1.65 (0.67-4.10)	0.27925	0.63 (0.19-2.14)	0.45946	0.76 (0.07-8.12)	0.81852
FGF10	rs13170645	1.64 (0.77-3.53)	0.20225	2.22 (0.99-4.96)	0.05220	0.80 (0.23-2.76)	0.72028	0.17 (0.02-1.62)	0.12284
SHH	rs208684	1.17 (0.67-2.06)	0.57643	1.66 (0.38-7.25)	0.49976	0.85 (0.23-3.11)	0.80129	NE	
FGF8	rs35344824	0.84 (0.17-4.28)	0.83857	NE		NE		NE	
TBX5	rs2295234	1.15 (0.65-2.05)	0.62324	0.88 (0.09-8.64)	0.91087	0.72 (0.21-2.42)	0.59472	2.57 (0.42-15.92)	0.30989
TBX5	rs1996821	1.11 (0.63-1.94)	0.72547	0.67 (0.25-1.78)	0.42028	1.82 (0.41-7.99)	0.42863	3.33 (0.66-16.74)	0.14363
TBX5	rs2236017	**2.00 (1.13-3.52)**	**0.01730**	1.21 (0.47-3.12)	0.69644	1.67 (0.38-7.32)	0.49866	1.22 (0.22-6.73)	0.81781
TBX5	rs7964303	1.22 (0.65-2.27)	0.53199	0.60 (0.28-1.32)	0.20301	0.84 (0.22-3.26)	0.80519	**5.62 (1.10-28.83)**	**0.03833**

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		Hispanic				Asian			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	**SNP**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**
TBX5	rs1946293	1.04 (0.56-1.95)	0.89241	0.65 (0.30-1.44)	0.28911	0.90 (0.23-3.52)	0.87958	1.80 (0.43-7.53)	0.42072
TBX5	rs3825214	1.46 (0.84-2.56)	0.18016	0.81 (0.25-2.63)	0.72551	0.71 (0.21-2.44)	0.58934	1.02 (0.21-4.97)	0.98253
TBX3	rs551510	0.98 (0.54-1.79)	0.95061	0.91 (0.42-1.97)	0.81800	0.59 (0.16-2.15)	0.42051	0.53 (0.13-2.24)	0.38894
GDF5	rs224333	1.16 (0.63-2.14)	0.64195	**2.29 (1.10-4.74)**	**0.02612**	7.37 (0.84-64.43)	0.07105	6.46 (0.68-61.15)	0.10372
SALL4	rs2179597	1.37 (0.74-2.55)	0.31715	2.25 (0.99-5.10)	0.05227	1.13 (0.32-4.07)	0.84784	2.72 (0.52-14.16)	0.23463
SALL4	rs6021451	1.37 (0.76-2.44)	0.29121	0.39 (0.05-3.26)	0.38508	1.21 (0.37-3.92)	0.75344	NE	
SALL4	rs6021435	0.91 (0.51-1.63)	0.75194	**2.76 (1.13-6.74)**	**0.02531**	0.73 (0.22-2.43)	0.61171	1.22 (0.19-7.90)	0.83302
Angiogenesis									
VEGFA	rs3025053	**3.55 (1.27-9.90)**	**0.01573**	NE		NE		NE	
NOS3	rs2853796	1.23 (0.66-2.30)	0.50594	**2.12 (1.03-4.38)**	**0.04151**	0.60 (0.16-2.20)	0.44124	2.08 (0.46-9.51)	0.34336
HIF1A	rs12434438	1.28 (0.72-2.28)	0.40445	0.92 (0.41-2.08)	0.84450	3.30 (0.85-12.84)	0.08495	**39.59 (3.90-401.49)**	**0.00185**
HIF1A	rs2301113	0.92 (0.51-1.66)	0.79354	0.97 (0.45-2.07)	0.93080	2.73 (0.69-10.86)	0.15295	**7.50 (1.53-36.71)**	**0.01290**

Abbreviations: OR=odds ratio, CI=95% confidence interval, NE= not estimable: no cases and/or no control infants with this genotype

*Results are presented for SNPs for which a nominally significant result ($p < 0.05$) was observed for at least one genotype comparison for any race/ethnic group.

†The reference group is the homozygous major genotype.

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Table V

Associations between Gene Variants and Limb Deficiencies by Phenotype*

		Transverse				Longitudinal				Intercalary			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	SNP	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value	OR (CI)†	p-value
Development													
WNT1	rs3754855	**0.69 (0.48-1.00)**	**0.04878**	0.80 (0.49-1.31)	0.38110	0.78 (0.54-1.12)	0.18057	0.90 (0.55-1.48)	0.67382	0.61 (0.31-1.21)	0.15760	0.83 (0.34-2.04)	0.68950
WNT1	rs893574	**1.96 (1.21-3.17)**	**0.00628**	NE		1.45 (0.86-2.44)	0.15940	6.59 (0.91-47.62)	0.06179	1.29 (0.48-3.46)	0.60790	NE	
WNT7A	rs11128667	**0.62 (0.43-0.89)**	**0.01062**	0.90 (0.49-1.67)	0.74725	1.26 (0.87-1.83)	0.21576	1.62 (0.89-2.95)	0.11116	1.52 (0.76-3.01)	0.23352	1.52 (0.48-4.82)	0.47932
WNT7A	rs12639607	**0.56 (0.36-0.87)**	**0.00980**	1.72 (0.70-4.21)	0.23639	1.02 (0.69-1.51)	0.90672	1.35 (0.50-3.69)	0.55357	0.98 (0.48-2.01)	0.95480	NE	0.98461
WNT7A	rs3762721	1.05 (0.70-1.57)	0.80697	**2.14 (1.19-3.83)**	**0.01091**	1.24 (0.84-1.84)	0.27747	1.17 (0.61-2.23)	0.64048	1.42 (0.71-2.85)	0.32719	0.73 (0.19-2.76)	0.64272
WNT7A	rs9863149	1.38 (0.95-1.98)	0.08699	**2.08 (1.25-3.45)**	**0.00456**	1.15 (0.80-1.64)	0.44649	0.89 (0.48-1.64)	0.71074	1.04 (0.55-1.98)	0.90648	0.81 (0.27-2.49)	0.71902
WNT7A	rs3796314	0.91 (0.60-1.39)	0.66608	**3.14 (1.27-7.73)**	**0.01290**	0.97 (0.64-1.47)	0.89413	0.42 (0.05-3.21)	0.40043	0.89 (0.40-1.98)	0.78212	1.87 (0.23-15.02)	0.55795
WNT7A	rs3796316	0.73 (0.49-1.08)	0.11041	**0.58 (0.35-0.94)**	**0.02724**	1.51 (0.98-2.32)	0.06216	1.27 (0.75-2.14)	0.36829	2.24 (0.96-5.22)	0.06077	1.81 (0.66-4.98)	0.25087
WNT7A	rs1946620	1.28 (0.86-1.91)	0.22005	**1.88 (1.18-2.99)**	**0.00820**	1.05 (0.72-1.54)	0.78725	0.86 (0.52-1.41)	0.54109	1.68 (0.83-3.44)	0.15213	0.73 (0.26-2.06)	0.54601
FGF10	rs10512852	**1.68 (1.15-2.46)**	**0.00773**	1.78 (0.58-5.46)	0.31636	0.96 (0.62-1.49)	0.86114	1.13 (0.32-3.95)	0.85078	1.23 (0.59-2.55)	0.58329	NE	
FGF10	rs10805683	**1.75 (1.24-2.49)**	**0.00167**	**2.25 (1.13-4.47)**	**0.02073**	**1.43 (1.00-2.03)**	**0.04806**	0.96 (0.39-2.33)	0.92155	1.52 (0.77-2.99)	0.22647	**4.03 (1.49-10.88)**	**0.00595**
FGF10	rs13170645	**1.55 (1.03-2.33)**	**0.03471**	**2.04 (1.26-3.32)**	**0.00390**	**1.57 (1.04-2.37)**	**0.03331**	**1.87 (1.13-3.09)**	**0.01426**	1.44 (0.67-3.07)	0.34954	1.59 (0.63-3.97)	0.32578
SHH	rs208684	0.84 (0.59-1.21)	0.35550	1.65 (0.85-3.19)	0.13688	0.95 (0.66-1.36)	0.76878	**2.30 (1.24-4.27)**	**0.00850**	1.78 (0.94-3.38)	0.07697	2.00 (0.56-7.09)	0.28334
FGF4	rs3740640	1.00 (0.66-1.51)	0.99894	0.48 (0.06-3.76)	0.48334	0.91 (0.59-1.39)	0.65593	1.39 (0.38-5.10)	0.61726	1.51 (0.74-3.09)	0.26275	**6.10 (1.20-31.00)**	**0.02915**
TBX5	rs2295234	**1.58 (1.10-2.28)**	**0.01377**	0.95 (0.28-3.27)	0.93907	1.13 (0.76-1.67)	0.54584	1.34 (0.49-3.61)	0.56877	1.20 (0.60-2.39)	0.60993	NE	
TBX5	rs12320320	0.98 (0.68-1.41)	0.90717	1.26 (0.68-2.32)	0.46271	0.83 (0.57-1.21)	0.32938	1.09 (0.59-2.04)	0.77828	**2.42 (1.26-4.64)**	**0.00773**	1.09 (0.24-4.91)	0.91023
TBX5	rs3782464	0.94 (0.65-1.35)	0.73351	1.01 (0.58-1.76)	0.97070	0.88 (0.61-1.28)	0.51096	1.03 (0.59-1.80)	0.91258	**3.14 (1.54-6.40)**	**0.00166**	1.95 (0.60-6.37)	0.27025
TBX5	rs1946293	1.10 (0.77-1.58)	0.59600	0.96 (0.56-1.64)	0.88188	0.72 (0.49-1.05)	0.08479	0.98 (0.60-1.62)	0.94618	**0.47 (0.24-0.93)**	**0.03096**	**0.27 (0.08-0.93)**	**0.03723**
TBX3	rs521082	0.89 (0.58-1.38)	0.60487	**3.55 (1.00-12.56)**	**0.04915**	1.02 (0.66-1.57)	0.92467	1.76 (0.36-8.64)	0.48348	1.00 (0.45-2.21)	0.99770	NE	
GREM1	rs17525764	1.03 (0.64-1.66)	0.89689	0.75 (0.17-3.34)	0.70727	**0.52 (0.29-0.96)**	**0.03582**	NE		1.40 (0.60-3.27)	0.43674	1.69 (0.21-13.30)	0.61878
SALL4	rs6021435	0.97 (0.68-1.39)	0.87835	1.06 (0.60-1.88)	0.83168	0.87 (0.59-1.27)	0.45936	**2.01 (1.23-3.28)**	**0.00501**	0.92 (0.48-1.76)	0.79161	0.53 (0.15-1.89)	0.32993
Angiogenesis													
VEGFA	rs3025010	1.03 (0.72-1.46)	0.88497	0.81 (0.44-1.47)	0.48490	1.27 (0.88-1.85)	0.20600	**1.75 (1.04-2.93)**	**0.03423**	0.56 (0.28-1.11)	0.09835	1.21 (0.50-2.93)	0.66626
NOS3	rs1800781	0.82 (0.54-1.25)	0.35305	0.85 (0.19-3.87)	0.83527	**0.62 (0.39-1.00)**	**0.04925**	1.90 (0.60-6.03)	0.27759	0.40 (0.14-1.15)	0.08839	3.45 (0.73-16.31)	0.11740
NOS3	rs3918186	0.76 (0.49-1.18)	0.22001	1.60 (0.33-7.83)	0.55883	0.93 (0.61-1.42)	0.73074	3.12 (0.90-10.86)	0.07325	**0.24 (0.07-0.79)**	**0.01864**	NE	

		Transverse				Longitudinal				Intercalary			
		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor		Heterozygous		Homozygous minor	
gene	**SNP**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**	**OR (CI)†**	**p-value**
NOS3	rs2853796	0.77 (0.53-1.13)	0.18076	0.89 (0.56-1.43)	0.64193	1.31 (0.85-2.02)	0.21917	**1.78 (1.09-2.92)**	**0.02124**	0.63 (0.32-1.24)	0.18334	0.59 (0.24-1.46)	0.25145
HIF1A	rs2284999	0.79 (0.52-1.22)	0.28997	0.85 (0.42-1.74)	0.66143	**1.71 (1.15-2.55)**	**0.00764**	1.81 (0.95-3.45)	0.07073	1.32 (0.64-2.74)	0.44998	0.81 (0.23-2.86)	0.74657

Abbreviations: SNP=single nucleotide polymorphism, OR=odds ratio, CI=95% confidence interval, NE=not estimable: no cases and/or no control infants with this genotype.

*Results are presented for SNPs for which a nominally significant result ($p < 0.05$) was observed for at least one genotype comparison for any limb deficiency phenotype.

†Odds ratios are adjusted for race/ethnicity; the reference group is the homozygous major genotype.

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