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Identification of Susceptibility Loci for Scoliosis in FIS Families With Triple Curves

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Abstract

The triple curve pattern (three lateral curvatures of equal severity) has been recognized as a distinct and unique clinical subtype of scoliosis. As part of a large study of familial idiopathic scoliosis (FIS), a subset of five families with a triple curve pattern (at least one member of each family having a triple curve) was evaluated to determine if this curve pattern was linked to any of the markers previously genotyped as part of the STRP-based previous linkage screen. Model independent linkage analysis (SIBPAL, v4.5) of the initial genomic screen identified candidate regions on chromosomes 6 and 10 when FIS was analyzed both as qualitative and quantitative traits in single- and multipoint linkage analyses. Additional fine mapping analyses of this subgroup with SNPs corroborated the findings in these regions ($P < 0.001$). These regions have been previously linked to FIS, however, this is the first time these regions have been implicated in a clinically well-defined subgroup and may suggest a unique genetic etiology for the formation of a triple curve.

Keywords

idiopathic scoliosis; triple curve; genomic; chromosomes; familial; loci; screen; identification; spine; genes

INTRODUCTION

Familial idiopathic scoliosis (FIS) is a structural lateral curvature of the spine that has its onset during puberty in otherwise normal children [Weinstein, 1994]. The condition occurs in approximately 2–3% of children, with the most severe curves occurring in 0.2–0.5% of (predominantly female) children [Kane, 1977; Weinstein and Ponseti, 1983]. Clinical and genetic studies support the hypothesis of genetic factors influential in its pathogenesis [Garland, 1934; Filho and Thompson, 1971; Bonati et al., 1976; Czeizel et al., 1978; Carr, 1990; Kesling and Reinker, 1997]. However, the mode of inheritance is still unclear. Studies to date have suggested autosomal dominant, multifactorial, or X-linked inheritance [Wynne-Davies, 1968; Cowell et al., 1972; Riseborough and Wynne-Davies, 1973; Axenovich et al., 1999]. A review of the recent literature suggests several candidate regions, including chromosome 6p, distal 10q, and 18q [Wise et al., 2000]; 17p11.2 [Salehi et al., 2002];

19p13.3 and 2q [Chan et al., 2002]; Xq23–26 [Justice et al., 2003]; 6p25–22, 6q14–16, 9q32–34, 16q11–q12 and 17p11–q11 [Miller et al., 2005]; 8q12 [Gao et al., 2007]; and 9q31.2–q34.2 and 17q25.3-qtel [Ocaka et al., 2008]. It is possible that several loci may play a role in the manifestation of the disorder and that the loci responsible may also vary from family to family, resulting in substantial clinical and genetic heterogeneity. Careful characterization of phenotypes within a study group is required to identify genetically homogenous groups of families that would enable us to delineate the complexity of this disorder.

Historically, a variety of classification systems for curve assessment have been developed in an effort to standardize patient groups to aid in clinical treatment, and predict potential outcomes [Schulthess, 1905; Cobb, 1948; Ponseti and Friedman, 1950; Stagnara and Queneau, 1953; James, 1954; Lange, 1956; Nash and Moe, 1969; Moe and Kettleson, 1970; Goldstein and Waugh, 1973; Travaglini, 1975; King et al., 1983; Asher, 1988; Bunch and Patwardhan, 1989; Cruickshank et al., 1989; Conrad et al., 1998; Lenke et al., 2002]. The first widely recognized classification of scoliosis was identified by Ponseti and Friedman in 1950 and is based on a five-curve classification. King et al. [1983] developed a principal means for classification of thoracic curves to select patients for surgery and vertebrae for optimal fusion. Both of these systems however, do not delineate a triple curve. The most recent method of classification, developed by Lenke et al. [2002], utilized a three-component assessment of the curve that includes curve type, a lumbar spine modifier, and a sagittal thoracic modifier. This classification system yields 42 possible types of curves and includes a triple curve category. Structural curvatures are described by their position in the spinal column, and their lack of flexibility. One or more than one of the curves may be considered major or primary in nature (the highest Cobb angle measurement on an anteroposterior standing spinal radiograph). The smaller curve(s) may then be considered minor; however, all of the curves lack flexibility, as determined through bending radiographs (Fig. 1).

The curves predominantly reported in the literature are single and double lateral curvatures. Triple curves, which involve three distinct structural lateral curves in the cervical, thoracic and lumbar vertebrae, are rare in comparison [Davidson et al., 2003]. Previous studies of classification systems and surgical outcomes reported that between 1.4 and 3% of cases examined have triple curves [Stokes et al., 1988; Conrad et al., 1998; Lenke et al., 2002; Davidson et al., 2003]. Cruickshank et al. [1989] reported that 27% of the patients in their study sample had a triple curve. In that study however, the assessment of curvatures examined addresses characterization of the upper thoracic region of the spine and indicated that many triple curves could have been diagnosed as double major curves if the small upper thoracic curvature was not considered.

The triple curve phenotype represents a small proportion of individuals with idiopathic scoliosis. Their scarcity makes the study of the natural history of these curvatures difficult. To date, studies suggest that when compared to other curve types, their course is generally benign and rarely progress to surgical intervention. In this study we focus on the triple curve phenotype and attempt to identify genetic variations that contribute to the overall process of scoliosis development and spinal stability.

MATERIALS AND METHODS

Sample and Ascertainment

In the original study, 202 families with at least two individuals with idiopathic scoliosis were ascertained and examined by a single orthopedic surgeon [Justice et al., 2003; Miller et al., 2005]. Written informed consent was obtained from all study participants, in accordance

with a protocol approved by the Johns Hopkins School of Medicine Institutional Review Board. In the original study the criteria for a diagnosis of scoliosis were history and physical examination consistent with a spinal curvature in the coronal plane, standing anteroposterior spinal radiographs exhibiting 10° curvature by the Cobb method with pedicle rotation and no congenital deformity [Shands and Eisberg, 1955; Kane, 1977; Armstrong et al., 1982]. If there was any historical evidence within the family of other genetic conditions, regardless of whether that member did or did not have scoliosis, the family was excluded from the current study sample [Shapiro et al., 1989; Boileau et al., 1993]. Additionally, individuals and families with secondary causes of idiopathic scoliosis were excluded from the study. The sample was consistent with previous epidemiological studies with respect to gender, curve type, and curve size as measured by the Cobb angle [Miller et al., 2001].

Triple Curves

In the present study, a subset of 5 families with 26 individuals was identified from the original study group (202 families) based on the identification of a triple curve phenotype. The triple curve pattern is three distinct scoliotic curves each measuring 10° , which are structural in nature, that is, curves that lack flexibility. Side bending AP radiographs were taken to determine the lack of flexibility of the curvatures, thus supporting the diagnosis of a triple curve pattern. Of these 26 individuals, 17 had scoliosis and 5 were classified as having a triple curve. To allow for the possibility of variable expressivity, any individual with scoliosis (regardless of type) was classified as affected for the purposes of this study. If an individual had more than one curve, the largest curve was used for curve measurement. The average curvature of all affected individuals was 32.7° and ranged from 11° – 60° .

Genomic Screen

Blood samples were obtained from all participants and genomic DNA was extracted using standard purification protocols [Ausubel, 1987; Moore and Dowhan, 2002; Fritsch et al., 1989]. A genomic screen for the 202 families was performed at the Center for Inherited Disease Research with a modified CHLC v.9 marker set consisting of 391 short tandem repeat markers [Miller et al., 2005].

Fine Mapping Short Tandem Repeat Polymorphisms

In the current study, additional short tandem repeat polymorphisms (STRPs) were genotyped in candidate regions suggestive of linkage. Database sources for STRPs (tetranucleotide and trinucleotides) included Marshfield Center for Medical Genetics (Marshfield, WI), the Ensembl Genome Browser, and the Centre d'Etude du Polymorphisme Humain (Paris, France). The markers were obtained as labeled map pairs (Research Genetics, Huntsville, AL). Polymerase chain reaction (PCR) amplification was performed in a $10\ \mu\text{l}$ reaction mix containing 40 ng of genomic DNA, a final concentration of $1 \times$ PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl_2 (Invitrogen), 0.25 mM dNTPs (Invitrogen), $0.24\ \mu\text{M}$ of forward primer and reverse primer (Research Genetics), and 1 U of Taq polymerase (Invitrogen). All primers were optimized for annealing temperature prior to amplification of samples. Annealing temperatures ranged from 55°C to 65°C . PCR conditions were 35 cycles at 95°C for 30 sec, the optimized annealing temperature for 1 min, and 72°C for 1 min, followed by one cycle at 72°C for 5 min.

Single Nucleotide Polymorphisms—Restriction Fragment Length Polymorphisms

All genotyped single nucleotide polymorphisms (SNPs) were identified through the National Center for Biotechnology Information (NCBI) dbSNP database (www.ncbi.nlm.nih.gov/SNP). SNPs were selected based on their location between markers d6s1043 and d6s501 on chromosome 6, with a reported minor allele frequency in dbSNP of

0.2; the SNP location being within a restriction enzyme cut site, and the number and size of fragments produced by a restriction enzyme. Eighteen SNPs formed the SNP map (1 SNP/150 kb) and PCR primers were chosen by the program Primer 3 [Rozen and Skaletsky, 2000]. PCR was performed in a 100 μ l reaction mix containing 120 ng of genomic DNA, a final concentration of 1 $\times$ PCR buffer (Invitrogen), 0.25 mM dNTPs (Invitrogen), 1.5 mM MgCl₂ (Invitrogen), 0.24 μ M of forward primer and reverse primer (Johns Hopkins University Genetics Resources CORE facility, Baltimore, MD), and 2 U of Hotstar *Taq*[®] DNA polymerase (Qiagen, Valencia, CA). All primers were optimized for annealing temperature prior to amplification by PCR. Annealing temperatures for the primers ranged from 55 to 65°C. PCR conditions were one cycle of 95°C for 15 min followed by 35 cycles of 95°C for 30 sec, the optimal annealing temperature for 1 min, and 72°C for 1 min, followed by one cycle at 72°C for 5 min. The PCR product was concentrated to 20 μ l using dry centrifugation. A restriction enzyme digestion was prepared in a 15 μ l reaction mix containing 5 μ l of the concentrated PCR product and a final concentration of 1 U of restriction enzyme (New England BioLabs, Beverly, MA), 1 $\times$ digestion buffer (New England BioLabs) and incubated according to the manufacturer's specifications. The digested product was separated out on a 2.5% Nusieve[®] 3:1 agarose gel, post-stained with 50 mg ethidium bromide. A 50-bp ladder (Invitrogen) was used to identify the different length fragments.

Single Nucleotide Polymorphisms—Allelic Discrimination With Taqman

Allelic discrimination was performed with the ABI Taqman method (Applied Biosystems, Inc., FosterCity, CA). A 5- μ l reaction mixture [20 ng of genomic DNA, 2.5 μ l of Taqman Universal PCR master mix (Applied Biosystems, Inc.), and 0.25 μ l of 20 $\times$ Assays-on-Demand SNP mix (Applied Biosystems, Inc.)] was prepared according to the manufacturer's specifications. PCR conditions were 95°C for 20 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. The PCR product then was analyzed for fluorescence with the ABI PRISM 7900 Sequence Detection System (Applied Bio-systems, Inc.).

Statistical Analyses

Allele frequencies for the 391 genomic screen markers were determined using FREQ [S.A.G.E., v4.5]. Familial relationships were verified using RELCHECK, which infers the most likely relationship between pairs of relatives by use of identity-by-descent sharing estimates [Boehnke and Cox, 1997; Broman and Weber, 1998]. Mendelian inconsistencies were determined using PEDCHECK [O'Connell and Weeks, 1995]. Non-systematic inconsistencies were removed from the data. Genetic and physical map distances for the STRPs were obtained from the University of California-Santa Cruz Human Genome browser (March 2004 release). Physical map distances for the SNPs were obtained from the NCBI dbSNP database (human build 35). Monomorphic SNPs and those with a *P*-value from an exact test for Hardy–Weinberg equilibrium less than 0.001 were discarded prior to linkage analysis.

Model-independent sib-pair linkage analysis was used to screen for linkage between the trait and each marker [SIBPAL, S.A.G.E., v4.5] on the subgroup of triples families (5 families, 27 individuals) for all autosomal markers. Sib-pair analyses were performed on 16 sib-pairs, of which 3 were discordant and 13 were concordantly affected. FIS was analyzed as a qualitative trait, where the threshold for affection was 10°, and as a quantitative trait, where the trait analyzed was the degree of lateral curvature. Skewness and kurtosis of the distribution of lateral curves were 0.41 and –1.08. The degree of lateral curvature was log transformed in an attempt to normalize the data. To corroborate these analyses, two additional linkage tests, the Whittemore and Halpern [1994] nonparametric linkage statistic

(NPL), and the Kong and Cox [1997] exponential model, were used to analyze the qualitative trait data, as implemented in the program MERLIN [Abecasis et al., 2002].

Prior to multipoint linkage analysis, pairs of SNPs were assessed for linkage disequilibrium (LD) using Haploview (version 3.2) [Barret, 2005]. A pair of SNPs were defined as being in high LD if $D' = 1.0$ and $r^2 \geq 0.4$. The most informative SNPs were retained from each cluster of SNPs in LD. This decreased the number of SNPs from 66 to 33 for chromosome 6 and from 34 to 16 for chromosome 10.

RESULTS

Genomic Screen

In this study, for the analyses of the STRP makers, a chromosomal region was considered significant if two or more adjacent markers had P -values less than 0.05. Five candidate regions were identified in the triples subgroup (chromosomes 6, 8, 10, 11, and 22, Table I). The regions on chromosomes 6, 8, and 11 were previously reported by Miller et al. [2005] in the original set of 202 families (1,198 individuals). In the triple curves subset, the most significant P -values were observed in regions on chromosome 6, D6S1056 ($P < 2 \times 10^{-10}$) and chromosome 10, D10S677 ($P < 5 \times 10^{-7}$). These regions span approximately 35 and 100 Mb, respectively. These regions were fine mapped with additional STRPs and SNPs. The areas on chromosomes 8 and 11 identified in this study are approximately 11 and 35 Mb upstream, respectively, from the regions reported by Miller et al. [2005]. The regions on chromosomes 10 and 22 were not reported as candidate regions in the previous analysis of our original population.

Fine-Mapping STRPs

Two additional STRPs within the chromosome 6 region and 4 additional STRPs within the chromosome 10 region were genotyped on all 27 individuals of the triples subgroup. Model-independent linkage analysis corroborated the regions initially identified by the genomic screen (Table I). The region on chromosome 6 in the triples subset was effectively narrowed to approximately 27 Mb. Linkage analysis on chromosome 10 revealed two possible regions (D10S2325-D10S1423 and D10S1765-D10S1239), each approximately 15 Mb in length. The distance between the two regions is approximately 70 Mb and includes the centromere. Based on the level of significance, the second region on chromosome 10 was considered a higher priority.

Fine-Mapping SNPs

Sixty-six SNPs between markers D6S1043 and D6S474 were genotyped on chromosome 6 (5 families, 27 individuals). Eighteen of the 66 SNPs on chromosome 6 were genotyped by RFLP-PCR. Of these, 6 out of 576 genotypes (1%) could not be determined. The remaining 48 SNPs were genotyped by the ABI Taqman method. Thirty-three of these SNPs were retained for analysis after running

Tagger on Haploview, version 3.2 [Barret, 2005]. Model independent linkage analysis revealed multiple consecutive SNPs significant at the 0.001 level (Fig. 2 and Table II). The NPL and Kong and Cox analyses corroborated these findings with multiple significant SNPs in the same region (Table II). When comparing the log of the inverse of the P -values obtained from all the SNPs and STRPs genotyped on chromosome 6, the SIBPAL, NPL, and Kong and Cox results were highly correlated with all the pairwise correlations being greater than 0.82 (Table III). The SNPs that are most significant in the qualitative Haseman–Elston analyses are the most significant SNPs for the NPL and Kong and Cox analyses.

Thirty-four SNPs between markers D10S1765 and D10S1239 were genotyped on chromosome 10 (5 families, 27 individuals). All 34 SNPs were genotyped using the ABI Taqman method. Sixteen SNPs were retained for analysis after running Tagger on Haploview, version 3.2 [Barret, 2005]. Model independent linkage analysis revealed multiple consecutive SNPs significant at $P < 0.001$ (Fig. 3 and Table IV). Linkage results obtained from the NPL and Kong and Cox analyses again corroborated these findings in the same region on chromosome 10 (Table IV). Pearson's correlation tests between the P -values obtained with the qualitative model and quantitative models as implemented in SIBPAL and the P -values obtained using NPL linkage and the Kong and Cox exponential model were again correlated (Table V), although not as highly correlated as the results on chromosome 6, with all the pairwise correlations being at least 0.61.

DISCUSSION

In this study, we identified a subgroup of families based on a triple curve phenotype from a large study group of FIS families. Genome-wide linkage analysis followed by SNP mapping of significant areas has identified two distinct chromosomal regions. These regions on chromosomes 6 and 10 were effectively narrowed to 1 and 7 Mb, respectively. In the original analysis (set of 202 families), linkage analysis identified the same region on chromosome 6. The area on chromosome 10 was unique to this phenotype in the current study group. Wise et al. [2000] identified regions on 6p, distal 10q, and 18q from a genome-wide linkage study in one pedigree. However, both the chromosomes 6 and 10 regions identified by Wise et al. [2000] are a considerable distance away from the areas we report in the current study.

Classification systems used for identification of curvature types are used in the determination of specific treatment modalities (e.g., bracing and/or surgery), because some curve types are amenable to specific brace types [Montgomery and Willner, 1989; Katz et al., 1997; Janicki et al., 2007]. The primary utilization of current classification schemes are the ability to identify which levels to be fused at surgery, and to be able to compare more specifically clinical study groups in relation to surgical intervention. While these classification systems have been very useful in the clinical domain, their relationship to the genetic factors believed to be contributing to this disorder is unknown. In an attempt to further minimize the genetic heterogeneity within this study group of FIS families, we identified and evaluated different curve phenotypes including kyphoscoliosis, left thoracic, and triple curves for their statistical significance.

Data from the triple curves subset corroborated the original finding on chromosome 6 and identified an additional region on chromosome 10. Due to the high significance of these areas within this phenotype, additional genotyping studies were performed. As with any statistical study, the results reported could be due to chance. Replication of these findings in an independent sample will be key to determining if this subgroup represents a distinct and homogenous clinical entity. However, the scarcity of families including individuals with triple curves will make independent replication difficult.

The clinical deformity of scoliosis is a lateral rotatory curvature of the spine, which appears in otherwise normal individuals. Studies involving the pathophysiology of the bone itself and surrounding soft tissue structures have not yet identified an element or factor that could be designated as causative. Avenues of investigation now include multiple biological pathways as potentially related to this disease process including the central and peripheral nervous system, endocrine and hormonal influences, bone development and maintenance, skeletal muscle and signaling pathways, embryological signaling, and growth plate dysfunction [Miller, 2007; Miller et al., 2006; Pourquie, 2008]. Several genes in the

candidate regions (Table VI) have been identified that may have relevance to the etiology of idiopathic scoliosis.

On chromosome 6, a more confined region than that on chromosome 10, Ephrin Receptor (EPHA7) and Fucosyltransferase 9 (FUT9) are of particular interest due to their involvement in the embryological and migrational aspects of the nervous system. Multiple studies have indicated the potential involvement of the nervous system as an instigating factor that ultimately results in the loss of spinal stability resulting in the lateral rotatory curvature. Several genes in the candidate regions (Table VI) have been identified that may have relevance to the etiology of idiopathic scoliosis. Examples include lysyl oxidase-like 4 gene (LOXL4), an enzyme that is involved in the cross-linking of collagen fibrils and insoluble elastic fibers within the extra cellular matrix, and 5-hydroxytryptamine receptor 7 (HTR7) gene, which regulates effects on osteoclast development and proliferation. Additional genes are involved in early embryonic signaling and development such as suppressor of fused (SUFU) and wingless type 8 (WNT8). SUFU maintains a critical part in neuronal tube development as part of the sonic hedgehog-signaling pathway. WNT8, as part of the WNT gene family, is involved in embryonic processes related to the spinal/ neuronal axis, and patterning of the early spinal cord. Despite the temporal disparity of the known action of these genes in embryonic development with that of the appearance of idiopathic scoliosis, underlying subtle aberrations in early regulatory processes hypothetically may lead to a later growth abnormality with hormonal signaling.

In conclusion, we have identified and narrowed candidate regions in a small subgroup of families with triple curves. Further analyses of these regions and sequencing of candidate genes will be required in order to identify genetic variants that may be associated with this disorder. Idiopathic scoliosis is the most common spinal deformity that is diagnosed in the skeletally immature individual. The definition of genetic markers associated with FIS will eventually allow the identification of those individuals with severely progressive disease. The immediate implication of the identification of an FIS gene is the development of a test for disease susceptibility and curve progression, with an ultimate goal of a test for accurate and early diagnosis. Once early diagnosis is available, more attention can be given to the development of non-surgical techniques that target spinal growth and development, with attention to responsiveness at the cellular level.

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FIG. 1.
Anteroposterior radiograph of one individual with a triple scoliotic lateral curvature of the spine.

FIG. 2.

P-plot of model-independent multipoint linkage analysis results for microsatellite markers and SNPs on chromosome 6. In this plot, the inverse of the log of the P -value is plotted against the location of each marker. The log scale gives more emphasis to highly significant results and less to results with a significance level closer to 1.0. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIG. 3.

P-plot of model-independent multipoint linkage analysis results for microsatellite markers and SNPs on chromosome 10. The p-plot is representative of the data as in Figure 2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I

Qualitative Model-Independent Linkage Analysis Results of Genomic Screen and Fine mapping STRP Markers

Marker	Mb ^a	Singlepoint <i>P</i> -values
d6s1O31	77.518	0.02888
d6s1O45	85.64	0.00015
d6s1O43 ^b	92.507	0.01774
d6s1O56	94.154	<0.00001
d6s501	97.938	0.00058
d6s475	100.711	0.00001
gatal64h01 ^b	102.53	0.00048
d6s1O21	104.781	0.00002
d6s474	112.986	0.00116
d8s1132	107.398	0.00029
d8s592	118.525	0.03001
d8s1128	128.664	0.04268
d10s1435	2.233	0.00015
d10s189	6.762	0.31750
d10s1412	9.304	0.49435
d10s2325	12.833	0.00077
d10s570 ^b	12.965	0.20341
d10s1653 ^b	15.718	0.01959
d10s1423	19.478	0.00738
d10s1225	64.425	0.08311
d10s1432	74.329	0.05106
d10s1658	85.765	0.26886
d10s1765	89.592	0.01023
d10s2470	92.355	0.00070
d10s185 ^b	95.178	0.00660
d10s677	95.954	<0.00001
d10s1239	103.186	0.01582
d11s1999	10.676	0.00001
d11s1981	17.042	0.01058
d22s689	27.181	0.00003
d22s685	32.92	0.02182
d22s683	34.838	0.04198
d22s44S	35.891	0.05376

^aMap positions from www.genome.ucse.edu.

^bFine mapping STRP markers.

TABLE II

Linkage Analysis of STRPs and SNPs on Chromosome 6

Marker	Mb ^a	Qualitative P-values Haseman-Elston			Quantitative P-values Haseman-Elston			Qualitative P-values		
		Singlepoint	Multipoint		Singlepoint	Multipoint		NPL multipoint	Kong and Cox multipoint	
d6s1043	92.51	0.01774	0.01871		0.01401	0.01532		0.0004	0.0002	
rs164545	94.13	<0.00001	<0.00001		0.0001	<0.00001		0.0003	0.0002	
d6s1056	94.15	<0.00001	<0.00001		<0.00001	0.00001		0.0003	0.0002	
rs549965	94.16	0.00391	<0.00001		0.00798	<0.00001		0.0004	0.0002	
rs599005	94.25	0.15837	<0.00001		0.21231	0.00001		0.0006	0.0005	
rs1386916	94.69	0.00203	<0.00001		0.00457	<0.00001		0.0006	0.0005	
rs1750604	94.96	0.00001	<0.00001		<0.00001	<0.00001		0.0006	0.0003	
rs220969	95.04	0.04236	<0.00001		0.01632	<0.00001		0.0006	0.0003	
rs9320497	96.13	<0.00001	<0.00001		<0.00001	0.00001		0.0008	0.0003	
rs2799642	96.47	0.00149	0.00001		0.00949	0.00002		0.0010	0.0007	
rs7752510	96.66	0.01096	0.00004		0.01563	0.00004		0.0012	0.0014	
rs9322788	96.81	0.22669	0.00017		0.16313	0.00008		0.0012	0.0014	
rs2205754	96.95	0.00004	0.00039		0.00001	0.00010		0.0010	0.0006	
d6s501	97.94	0.00058	0.00007		0.00068	0.00004		0.0002	0.0001	
d6s475	100.72	<0.00001	0.00002		0.00005	0.00005		0.0005	0.0003	
gata164h01	102.53	0.00048	0.00008		0.00171	0.00017		0.0009	0.0007	
rs2518271	102.55	0.44689	0.00008		0.39512	0.00017		0.0009	0.0007	
rs2399802	103.00	0.12162	0.00008		0.13523	0.00017		0.0009	0.0007	
rs537534	103.02	0.00027	0.00008		0.00087	0.00017		0.0009	0.0007	
rs1041927	103.54	0.00002	0.00008		0.00042	0.00016		0.0009	0.0007	
rs9377547	104.18	0.00179	0.00008		0.00321	0.00016		0.0008	0.0006	
rs926275	104.56	0.00001	0.00008		0.00004	0.00016		0.0009	0.0007	
rs6571154	104.57	0.00096	0.00008		0.00103	0.00016		0.0009	0.0007	
d6s1021	104.78	0.00002	0.00005		0.00002	0.00011		0.0008	0.0006	
rs167539	105.52	0.00186	0.00003		0.00473	0.00006		0.0007	0.0006	
rs9399916	106.08	0.00025	0.00003		0.00017	0.00005		0.0007	0.0005	
rs1984224	106.64	0.01722	0.00003		0.03614	0.00005		0.0006	0.0005	
rs11153009	107.13	0.00001	0.00006		0.00004	0.00008		0.0040	0.0010	

Marker	Mb ^a	Qualitative <i>P</i> -values		Haseman-Elston		Quantitative <i>P</i> -values		Haseman-Elston		Qualitative <i>P</i> -values	
		Singlepoint	Multipoint	Singlepoint	Multipoint	Singlepoint	Multipoint	Singlepoint	Multipoint	NPL multipoint	Kong and Cox multipoint
rs7754744	107.66	0.02521	0.00066	0.02696	0.00061	0.0200	0.0300				
rs1625630	108.14	0.33741	0.00042	0.29866	0.00046	0.0400	0.0500				
rs217529	108.61	0.02583	0.0013	0.03316	0.00165	0.0500	0.0700				
rs2802290	109.01	0.00184	0.00133	0.00386	0.00165	0.0500	0.0700				
rs9398182	109.37	< 0.00001	0.00133	< 0.00001	0.00165	0.0500	0.0700				
rs1358997	109.81	0.00013	0.00134	0.00013	0.00166	0.0500	0.0700				
rs4072342	110.23	0.38271	0.00135	0.46401	0.00167	0.0500	0.0700				
rs9374172	110.84	0.45326	0.0013	0.52152	0.00162	0.0500	0.0700				
rs1543990	111.04	0.01483	0.00129	0.02553	0.0016	0.0500	0.0700				
rs20719626	112.12	0.00173	0.00128	0.00015	0.00159	0.0600	0.0700				
rs1050348	112.60	0.48402	0.00125	0.56811	0.00156	0.0600	0.0700				
d6s474	112.99	0.00116	0.00123	0.00198	0.00154	0.0600	0.0700				

P-values <0.01 shown in bold.

^aMap positions from www.genome.ucsf.edu.

TABLE III

Pairwise Pearson's Correlation Coefficients of Minus the Log of the *P*-Values for SNPs and STRPs on Chromosome 6

	SIBPAL qualitative	SIBPAL quantitative	NPL	Kong and Cox
SIBPAL qualitative	1.0000	0.9881	0.8362	0.8057
		<0.0001	<0.0001	<0.0001
SIBPAL quantitative		1.0000	0.8457	0.8265
			<0.0001	<0.0001
NPL			1.0000	0.9201
				<0.0001
Kong and Cox				1.0000

r^2 and significance of the r^2 are listed.

TABLE IV

Linkage Analysis Results of STRPs and SNPs on Chromosome 10

Marker	Mb ^a	Qualitative <i>P</i> -values Haseman-Elston			Qualitative <i>P</i> -values Haseman-Elston			Quantitative <i>P</i> -values	
		Singlepoint	Multipoint	Multipoint	Singlepoint	Multipoint	Multipoint	NPL multipoint	Kong and Cox multipoint
d10s1765	89.59	0.01023	0.01056	0.01056	0.01796	0.01817	0.02000	0.02000	0.02000
rs2296545	90.33	0.03429	0.00359	0.00359	0.01248	0.00523	0.02000	0.02000	0.01300
rs2274878	91.09	0.02727	0.00133	0.00133	0.01844	0.00143	0.01500	0.01500	0.01100
rs962524	91.52	< 0.00001	0.0054	0.0054	< 0.00001	0.0061V	0.00700	0.00700	0.00500
d10s2470	92.33	0.0007	0.00032	0.00032	0.00065	0.00023	0.00300	0.00300	0.00300
rs12412496	92.54	< 0.00001	0.00032	0.00032	< 0.00001	0.00023	0.00200	0.00200	0.00300
rs7913826	93.57	0.03252	0.00032	0.00032	0.05268	0.00022	0.00200	0.00200	0.00200
rs10748585	94.67	0.03112	0.00032	0.00032	0.06278	0.00022	0.00200	0.00200	0.00200
10s185	95.18	0.00660	0.00320	0.00320	0.01660	0.00022	0.00200	0.00200	0.00200
rs1555870	95.51	0.05395	0.00032	0.00032	0.04093	0.00022	0.00150	0.00150	0.00200
d10s677	95.95	< 0.00001	0.00032	0.00032	<0.00001	0.00022	0.00150	0.00150	0.00200
rs1408820	96.00	0.35869	0.00032	0.00032	0.32229	0.00022	0.00150	0.00150	0.00200
rs7898759	96.79	0.02461	0.00032	0.00032	0.02664	0.00022	0.00200	0.00200	0.00200
rs732102	97.18	0.00017	0.00032	0.00032	0.00285	0.00022	0.00200	0.00200	0.00200
rs12571884	98.37	0.35603	0.00033	0.00033	0.31990	0.00022	0.00200	0.00200	0.00200
rs7899632	99.99	< 0.00001	0.0003	0.0003	< 0.000001	0.00021	0.00200	0.00200	0.00200
rs7094763	100.49	0.13853	0.03256	0.03256	0.03882	0.02112	0.00003	0.00003	0.00013
rs4919438	102.00	< 0.00001	0.00247	0.00247	< 0.00001	0.00059	0.00003	0.00003	0.00012
rs1361265	102.86	0.44611	0.00267	0.00267	0.39126	0.00065	0.00003	0.00003	0.00012
rs946327	102.88	0.0015	0.00268	0.00268	0.00045	0.00065	0.00003	0.00003	0.00012
d10s1239	103.19	0.01582	0.00291	0.00291	0.03520	0.00073	0.00004	0.00004	0.00012
d10s1237	116.11	0.31373	0.34653	0.34653	0.31313	0.31709	0.03000	0.03000	0.04000

P-values <0.01 shown in bold.

^aMap positions from www.genome.ucsf.edu.

TABLE V

Pairwise Pearson's Correlation Coefficients of Minus the Log of the Inverse of the P -Values for SNPs and STRPs on Chromosome 10

	SIBPAL qualitative	SIBPAL quantitative	NPL	Kong and Cox
SIBPAL qualitative	1.0000	0.9844	0.6109	0.6564
		<0.0001	0.0001	<0.0001
SIBPAL quantitative		1.0000	0.6906	0.7363
			<0.0001	<0.0001
NPL			1.0000	0.9886
				<0.0001
Kong & Cox				1.0000

r^2 and significance of the r^2 are listed.

TABLE VI**Candidate Genes on Chromosomes 6 and 10**

Candidate gene	Chromosome	Description
EPHA7	6	Ligand involved in the repulsion of axonal growth cones and migrating cells
FUT9	6	Synthesizes the Lewis X oligosaccharide is expressed in organ buds during embryogenesis and is developmentally regulated. Functions in cell to cell interaction during neuronal development
MANEA	6	Secretory pathway glycosylation enzyme found in mitochondria
CHUK	10	Essential for NFKB activation during limb and craniofacial development
CRTAC1	10	An extracellular matrix protein expressed in bone, cartilage, and cultured chondrocytes
CSPG6	10	Chondroitin sulfate proteoglycan
FBXW4	10	Involved in signaling pathways crucial for normal limb development/split-hand/foot malformation
FER1L3	10	Connected to muscular dystrophies
HABP2	10	Glycosaminoglycan that is present in the extracellular matrix, connective tissue, cartilage, bone marrow, and synovial fluid
HTR7	10	Serotonin receptor, regulatory effects on osteoclast development
KAZALD1	10	Promotes proliferation of osteoblastic cells
LBX1	10	Expressed during embryogenesis, expression restricted to developing CNS and muscles
LOXL4	10	Cross-linking of collagen fibrils and insoluble elastic fibers in ECM, expressed in hypertrophic/calcified chondrocytes/growth plates
NFKB2	10	Bone development/knockout mouse model of osteopetrosis due to a defect in osteoclast differentiation
NRAP	10	Giant myofibrillar proteins found within the sarcomeres of skeletal muscle
PLCE1	10	Initiate a cascade of intracellular responses that result in cell growth and differentiation and gene expression
SEMA4G	10	Belongs to the semaphorin family, axonal guidance, and development in the A/P axis between the dorsal and ventral margins of the trunk and tail (zebrafish)
SFRP5	10	Member of “frizzled” family of proteins/function as receptors for WNT genes
SLIT1	10	Play a critical role in central nervous system midline formation
SUFU	10	Part of the Sonic Hedgehog signaling pathway/neural tube development
TLL2	10	Structurally related to BMP-I/functions in ECM formation
WNT8	10	WNT genes encode intercellular signaling glycoproteins that play important roles in key processes of embryonic development such as mesoderm induction, specification of the embryonic axis, and patterning of the central nervous system, spinal cord, and limbs