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Hypospadias and genes related to genital tubercle and early urethral development

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Abstract

Purpose—We determined whether variants in genes associated with genital tubercle (the anlage for the penis) and early urethral development were associated with hypospadias in humans.

Materials and Methods—We examined 293 relatively common tagSNPs in *BMP4, BMP7, FGF8, FGF10, FGFR2, HOXA13, HOXD13, HOXA4, HOXB6, SRY, WT1, WTAP, SHH, GLI1, GLI2*, and *GLI3*. The analysis included 624 cases (81 mild, 319 moderate, 209 severe, 15 undetermined severity) and 844 population-based non-malformed male controls born in California from 1990-2003.

Results—There were 28 SNPs for which any of the comparisons (i.e., overall or for a specific severity) had a p-value <0.01. The homozygous variant genotypes for four SNPs in *BMP7* were associated with at least 2-fold increased risk of hypospadias, regardless of severity. Five SNPs for *FGF10* were associated with 3- to 4-fold increased risks, regardless of severity; for four of them, results were restricted to whites. For *GLI1*, *GLI2* and *GLI3*, there were 12 associated SNPs but results were inconsistent by severity and race-ethnicity. For *SHH*, one SNP was associated with 2.4-fold increased risk of moderate hypospadias. For *WT1*, six SNPs were associated with approximately 2-fold increased risks, primarily for severe hypospadias.

Conclusions—This study provides evidence that SNPs in several genes that contribute to genital tubercle and early urethral development are associated with hypospadias risk.

Keywords

hypospadias; genes; genital tubercle

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INTRODUCTION

Hypospadias is a common congenital malformation in which the urethral meatus is on the ventral side of the penis [1, 2]. Familial patterns indicate that genetic factors substantially contribute to its etiology [3].

Early development of the external genitalia and urethra depends on the formation and outgrowth of the genital tubercle, the anlage for the penis. This early stage is largely androgen-independent and precedes sexual differentiation. Experimental studies support the contribution of many transcription factors and signaling molecules to this process, including Wilms tumor 1, sonic hedgehog, homeobox proteins, bone morphogenetic proteins, fibroblast growth factors, and Gli transcription factors [4-9]. Expression of the sex-determining region Y gene (*SRY*) and its direct target, *SOX9*, is critical to the initiation of the next phase, which is androgen-dependent and involves sexual differentiation and development of the penis and testes [10]. Previous studies have investigated the association of hypospadias with genes that contribute to these events. A greater frequency of variants in cases than controls has been reported for *BMP4, BMP7, HOXA4, HOXA6*, and *WT1* but not *SRY* or *SOX9* among Chinese males [11, 12], *FGF8* and *FGFR2* but not *BMP7* or *FGF10* among Swedish males [13], and one study did not observe differences among German males for *HOXA13* or *WTAP* [14]. Severity of the phenotypes varied. These studies have primarily involved sequencing, among few subjects. [15].

Our hypothesis was that variants in genes associated with genital tubercle and early urethral development are associated with human hypospadias. We examined close to 300 relatively common variants in a large population of California male infants in the following genes, given that they have been the subject of previous genetic association studies in humans: *BMP4, BMP7, FGF8, FGF10, FGFR2, HOXA13, HOXD13, HOXA4, HOXB6, SRY and SOX9, WT1, and WTAP*. We also examined *SHH*, *GLI1*, *GLI2*, and *GLI3* (Table 1).

MATERIALS AND METHODS

The study population included male infants born from 1990-2003 to mothers who were residents of eight California Central Valley counties and from 1990-1997 to mothers who were residents of Los Angeles, San Francisco, and Santa Clara counties, reflecting counties where case ascertainment was actively conducted by the California Birth Defects Monitoring Program (CBDMP) by reviewing medical records at hospitals and genetic centers [16].

Cases were classified as mild (meatus was limited to the coronal or glanular penis, British Pediatric Association [BPA] codes 752.605, 752.625), moderate (meatus on the penile shaft, BPA 752.606, 752.626), or severe (meatus at the peno-scrotal junction or perineal area, BPA 752.607, 752.627). Assignment of severity was finalized based on review by a medical geneticist (EJL or Dr. Cynthia Curry) [17]. Cases for which the anatomical position was not sufficiently described ("not-otherwise-specified," BPA codes 752.600, 752.620) were excluded. Cases classified as having a known single gene disorder or chromosomal abnormality were excluded. Mild cases without chordee (BPA752.605) were not ascertained

by CBDMP except in 2004; thus, mild cases are under-represented and primarily those with chordee [17].

The underlying study population included 1,246,172 non-malformed live born male infants eligible for control selection. We randomly selected 931 with available bloodspots (the DNA source), in proportion to the underlying birth population for each year, to give an approximate 2:1 ratio of controls to cases from Central Valley counties and a 1:1 ratio from other counties. The ratio differed due to the presence of a secondary on-going study in the Central Valley.

Covariates were from birth certificates: maternal race-ethnicity, education, age, and parity; plurality; and infant birthweight and gestational age at delivery. In total, 667 (88% of eligible) cases and 931 (93% of eligible) controls were available for genotyping.

Genomic DNA was extracted from dried bloodspots using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies Madison, WI), and 10 ng genomic DNA was then used for whole genome amplification (Qiagen Repli-g® kit). TagSNPs that assay known common SNPs either directly or indirectly via linkage disequilibrium among measured and unmeasured SNPs were selected (http://gvs.gs.washington.edu/GVS/). The program provided tagSNPs that cover common variation at r^2>0.80 across each candidate gene for a “cosmopolitan” population, including Hispanics. TagSNPs with minor allele frequencies (MAF) >10% were selected. For *FGF10* and *SRY* we selected tagSNPs with MAF>1% because none had MAF>10%. For *GLI2* and *GLI3* we excluded the Yoruban (YRI) population from tagSNP selection to reduce the number of SNPs (both genes initially produced >100 tagSNPs). For *FGFR2*, we limited SNP selection to 2 non-intronic tagSNPs due to limited assay space. SNPs were genotyped using a custom multiplex Illumina GoldenGate assay.

We started with 324 SNPs. We excluded 19 for which the data indicated poor clustering of results and one with a call rate <90%. We also excluded 126 subjects (41 cases, 85 controls) with sample call rates <90%, leaving 626 cases and 846 controls for analyses. We further excluded 11 SNPs with p<0.01 for Hardy-Weinberg test of equilibrium among non-Hispanic white or Hispanic controls, leaving 293 for analysis. The single SNP we measured for *SOX9* was excluded based on the Hardy-Weinberg test.

Following our previous work, we genotyped 106 ancestry informative marker (AIM) SNPs to discriminate Native American, African, and European ancestry [18-21], and we used Structure 2.1 to estimate individual ancestry estimates (IAE) [22, 23]. Four SNPs were excluded that had call rates <90%. Structure was run using the admixture model with unlinked markers, with 50,000 burn-in iterations and 50,000 further iterations. Structure provided variables reflecting the proportions of Native American, African and European ancestry for each subject. Given that the three proportions sum to one, analyses included only two (Native American and African).

We used logistic regression to compare homozygous and heterozygous variant genotypes with homozygous wildtype (the more frequent allele among controls was designated as wildtype). We considered the presence of population stratification by examining models

restricted to self-identified non-Hispanic white and Hispanic subjects that contained product terms to estimate interaction. For SNPs for which the overall p-value for the product term was <0.10 (n=21), we focused on stratified results. We conducted analyses of all cases grouped together as well as separately by severity.

For the **9** genes for which there were >5 SNPs (*BMP7, FGF8, FGF10, HOXB6, GLI1, GLI2, GLI3, WT1, WTAP*), we examined haplotypes. We used Haploview 4.2 to determine the LD structure and to define haplotype blocks and their frequencies based on all subjects' genotypes [24]. The most common haplotype was the reference. Maximum likelihood estimates of odds ratios (OR) and their corresponding 95% confidence intervals (CI) were calculated from logistic regression models to estimate relative risks.

We also evaluated genetic risk scores created by combining high-risk SNPs. For each individual we counted the number of genes in which they carried an associated variant ($p<0.01$). For variants with ORs<1, the reference genotype (homozygous wildtype) was scored as the risk genotype. We calculated scores overall and separately by severity (applying the p-value criterion within each group, such that a somewhat different set of variants was scored within each group). We then estimated ORs and 95% CIs associated with the risk scores, using logistic regression.

All ORs were adjusted for the two ancestral proportion variables and for maternal residence in the Central Valley due to the differing case-control ratio based on this variable inherent to the study design. In addition, non-stratified results were adjusted for maternal race-ethnicity (Hispanic, non-Hispanic white, or other). Two cases and two controls had missing race-ethnicity, such that SNP-based analyses included 844 controls and a maximum 624 cases (81 mild, 319 moderate, 209 severe, 15 undetermined).

The study was approved by the California Health and Human Services Agency Committee for the Protection of Human Subjects and Institutional Review Boards at Stanford University and Children's Hospital Oakland.

RESULTS

Comparing case versus control mothers, the former were more likely non-Hispanic white, more highly educated, older, and nulliparous (Table 2). Cases were more likely to be low birthweight and delivered before 37 weeks of gestation.

There were 28 SNPs for which any comparison had $p<0.01$ (Table 3). (Results for other SNPs are available in a supplementary table). The homozygous variant genotypes for four SNPs in *BMP7* were associated with at least 2-fold increased risk of hypospadias, regardless of severity. Five SNPs for *FGF10* were associated with 3-4-fold increased risks, regardless of severity; for four of these, results were restricted to whites. For *GLI1*, six SNPs were associated: three with reduced risk and three with increased risk, primarily among moderate cases. For *GLI2*, two SNPs were associated with 2-fold increased risks for moderate phenotypes, but only among whites, and one SNP was associated with 2-fold increased risk, but only for moderate hypospadias (severe phenotypes were also associated with increased risk for two of the SNPs, albeit $p>0.01$). For *GLI3*, one SNP was associated with increased

risk of mild, one with moderate, and one with severe hypospadias. For *SHH*, one SNP was associated with increased risk of moderate hypospadias. For *WT1*, six SNPs were associated with approximately 2-fold increased risk, primarily for severe hypospadias.

The similarity in results across multiple SNPs for certain genes reflected high linkage disequilibrium in some instances but not others. For example, for *BMP7* the pair-wise R-squared values for three of the four SNPs ranged from 0.83 to 0.95 (the fourth SNP, rs607007, was rare). For the other genes, the ranges were as follows: 0.43-0.97 for *FGF10*; 0.54-0.91 for *GLI1*; 0.28-0.65 for *GLI2*; 0.03-0.09 for *GLI3*; and 0.48-0.86 for *WT1*, with the exception of rs12293750, for which there was only one homozygous variant subject (data not shown).

Haplotype analyses, which included all cases as one group, produced two results with $p<0.01$. For a block in *GLI3* that included rs2237421, rs2237420, rs1527499 and rs3801165, the OR for the ACTG haplotype versus ACGG was 1.5 (95% CI 1.1, 2.0), $p=0.0079$. For a block in WTAP that included rs963800, rs2758313, rs2842972, rs4709364, rs3822849 and rs1440, the OR for the CCCCTG versus CCTCTG haplotype was 3.3 (95% CI 1.5, 7.3), $p=0.002$.

Results for the risk score analysis reflect the increase in risk due to having risk-associated SNPs in multiple genes (Table 4). As expected, a higher number of risk genes corresponded with higher ORs. For example, among severe cases a score of two or three was associated with at least a 3-fold increased risk, whereas a score of one was associated with a 1.6-fold increased risk.

DISCUSSION

SNPs in several genes that contribute to genital tubercle and early urethral development were associated with hypospadias risk, including *BMP7, FGF10, GLI* transcription factors, *SHH* and *WT1*. Results did not suggest an association with *BMP4, FGF8, FGFR2, SRY* or *WTAP*. This study included a more in-depth investigation of variants in these genes than previous studies, in a large, racially-ethnically diverse study population.

Experimental studies indicate that bone morphogenetic proteins and fibroblast growth factors contribute to genital tubercle development [4, 6]. Two studies have examined their contribution to hypospadias in humans. Sequence variations in *BMP4* were reported among three cases and in *BMP7* among six cases, out of 90, and none among 190 controls [11]. Among 60 Swedish boys with familial, isolated hypospadias, four had variants in *FGF8* and seven had variants in *FGFR2*, which were not observed among 96 controls [13]. Several *FGFR2* relatively frequent polymorphisms were observed but not different among cases versus controls. No sequence variations were reported in *FGF10* or *BMP7* [13]. In the current study, SNPs in *BMP4, FGF8,* and *FGFR2* were not associated with hypospadias, whereas, several SNPs in *BMP7* and *FGF10* were associated with at least 2-fold increased risks of hypospadias, regardless of severity. Findings for *FGF10* were predominately among non-Hispanic whites.

SHH contributes to epithelial-mesenchymal interactions and patterning in the genital tubercle [25], and *Shh* knockout mice have genital tubercle agenesis [9]. Gli transcription factors are encoded by three genes with overlapping function (*GLI1-3*), which are regulated by *SHH* [25]. GLI genes are associated with limb and craniofacial development, which involve developmental processes related to tissue patterning that may apply to urethral development [25]. Gli2 mutant mice have defective urethral formation [9, 25]. To our knowledge, the current study represents the first investigation of *GLI1-3* or *SHH* and hypospadias in humans. Our study provided some evidence of an association for selected variants in these genes, but results were inconsistent across phenotypes and race-ethnicity.

WT1 is a zinc-finger transcription factor that contributes to normal development of the genitourinary system [26, 27]. *WTAP* contributes to *WT1* function [28]. Sequence variations in *WT1* were reported in three of 90 Chinese cases and zero of 276 controls [12], and zero of 35 Swedish cases [29]. Variants in *WT1* were reported among six of 80 severe hypospadias cases with cryptorchidism; all six eventually developed Wilms tumor or nephropathy [30]. No variants were observed among 70 cases without cryptorchidism. Utsch et al. sequenced the exons of *WTAP* [14]. They observed two variants and stated that their frequency was not different between cases and controls. In the current study, a *WTAP* haplotype and several SNPs in *WT1* were associated with hypospadias.

Experimental evidence also suggests that homeobox genes contribute to early genital tubercle development [4, 5]. *Hoxa13* knockout mice have hypospadias [4], and *Hoxa13* mutants have altered androgen receptor expression [4]. In humans, mutations in *HOXA13* cause hand-foot-genital syndrome, which includes hypospadias [5]. However, no sequence variants in *HOXA13* were observed among 37 cases with hypospadias [14]. Variants in *HOXA4* were observed among three subjects and in *HOXA6* among two subjects, among 90 total, and none among controls [11]. The current study did not provide support for an association of common variants in several homeobox genes with hypospadias.

Our study is strengthened by its size, population-based controls, ancestry informative markers, and inclusion of multiple genes. We chose to highlight results that met a relatively modest criterion for statistical significance ($p<0.01$) rather than conducting formal correction for multiple testing, given that our study focused on candidate genes. Our approach minimizes Type II errors (false negatives). However, we acknowledge that the trade-off is an increased possibility of false positive results. As such, we emphasize the need for replication of our findings in additional study populations. Our approach of investigating tagSNPs seemed appropriate, given that it captures the majority of genetic variation and is cost-efficient, and that minimal examination of the studied genes in humans preceded the current study. However, most tagSNPs are intronic and have no known functional consequences. Two exonic SNPs are included in Table 3 but are non-synonymous variants (rs2228226 in *GLI1* and rs16754 in *WT1*). Thus, observed associations are likely driven by linkage disequilibrium with other less common unmeasured variants and merit further genetic inquiry. Elucidation of underlying causal variants could be useful for genetic counseling purposes or for directing mechanistic studies. Also of note, we were able to explore results by phenotypic severity, but sample size for some phenotype-specific results were limited, especially for mild cases. Under-ascertainment of mild cases or

misclassification of severity are potential limitations but unlikely to be responsible for our results.

CONCLUSIONS

This study found substantial evidence for an association of hypospadias with genes involved in genital tubercle development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Genes included in analyses.

Gene name	Gene symbol	Role	Number of SNPs analyzed (total=293)
Bone morphogenetic protein 4	*BMP4*	GT signaling cascade	3
Bone morphogenetic protein 7	*BMP7*	GT signaling cascade	62
Fibroblast growth factor 10	*FGF10*	GT signaling cascade	25
Fibroblast growth factor 8	*FGF8*	GT signaling cascade	7
Fibroblast growth factor receptor 2	*FGFR2*	GT signaling cascade	1
GLI family zinc finger 1	*GLI1*	GT signaling cascade,	7
GLI family zinc finger 2	*GLI2*	GT signaling cascade	45
GLI family zinc finger 3	*GLI3*	GT signaling cascade	91
Homeobox A13	*HOXA13*	GT signaling cascade	3
Homeobox D13	*HOXD13*	GT signaling cascade	2
Homeobox A4	*HOXA4*	GT signaling cascade, penile skin deveopment	3
Homeobox B6	*HOXB6*	GT signaling cascade, penile skin development	6
Sonic hedgehog	*SHH*	GT signaling cascade	3
Sex determining region Y	*SRY*	Initiation of sexual differentiation stage	1
Wilms tumor 1	*WT1*	Early GT development	27
Wilms tumor 1 associated protein	*WTAP*	Early GT development	7

GT = genital tubercle

Table 2

Descriptive characteristics of cases with hypospadias (n=626) and non-malformed controls (n=846).

	Percent of Controls (n)	Percent of Cases (n)
Maternal race-ethnicity		
White	31 (258)	44 (274)
Hispanic	52 (439)	34 (215)
Others	17 (147)	22 (135)
Unknown	<1 (2)	<1 (2)
Maternal education		
< High school	39 (330)	25 (159)
High school	31 (265)	27 (170)
> High school	29 (244)	47 (295)
Unknown	<1 (7)	<1 (2)
Maternal age		
< 25 years	46 (393)	30 (185)
25-34 years	43 (360)	52 (329)
35 or more years	11 (93)	18 (112)
Number of previous live births		
0	36 (308)	52 (328)
1	33 (275)	26 (162)
≥ 2	31 (263)	21 (134)
Unknown	0 (0)	<1 (2)
Infant birthweight		
≤ 2500 g	5 (42)	30 (190)
> 2500 g	95 (804)	70 (436)
Gestational age at delivery		
< 37 weeks	7 (60)	23 (143)
≥ 37 weeks	89 (750)	74 (462)
Unknown	4 (36)	3 (21)
Maternal residence in Central Valley		
No	45 (381)	63 (392)
Yes	55 (465)	37 (234)

Table 3

Association of hypospadias with selected SNPs.*

Gene, SNP (Alleles)	MAF (Con-trols)	Geno-type	No. Controls	No. Cases	OR (95% CI) All Cases	P	No. Mild Cases	OR (95% CI) Mild Cases	P	No. Moderate	OR (95% CI) Moderate Cases	P	No. Severe Cases	OR (95% CI) Severe Cases	P
BMP7 rs6070007 (C:A)	0.007	CC	833	607	Reference		81	Reference		306	Reference		205	Reference	
		AC	10	17	2.4 (1.0 - 5.4)	0.041	0			13	**4.2 (1.7 - 10.5)**	**0.002**	4	2.2 (0.7 - 7.7)	0.201
		AA	1	0	NC		0			0	NC		0	NC	
rs6127978 (A:G)	0.213	AA	517	380	Reference		51	Reference		192	Reference		129	Reference	
		AG	283	198	1.0 (0.8 - 1.3)	0.895	24	1.1 (0.6 - 1.8)	0.844	109	1.1 (0.8 - 1.5)	0.467	60	0.8 (0.6 - 1.2)	0.350
		GG	37	45	1.9 (1.2 - 3.0)	**0.009**	6	2.3 (0.8 - 6.3)	0.109	17	1.5 (0.8 - 2.8)	0.217	20	2.2 (1.2 - 4.0)	0.011
rs6127980 (G:A)	0.197	GG	539	405	Reference		54	Reference		206	Reference		136	Reference	
		AG	277	180	0.9 (0.7 - 1.2)	0.584	21	1.0 (0.6 - 1.8)	0.95	100	1.0 (0.8 - 1.4)	0.870	54	0.7 (0.5 - 1.1)	0.114
		AA	28	38	**2.2 (1.3 - 3.8)**	**0.003**	6	3.8 (1.3 - 11.2)	0.014	13	1.6 (0.8 - 3.3)	0.213	18	**2.6 (1.4 - 5.1)**	**0.004**
rs6127985 (G:A)	0.207	GG	528	380	Reference		51	Reference		192	Reference		129	Reference	
		AG	280	202	1.1 (0.8 - 1.3)	0.588	24	1.1 (0.6 - 1.9)	0.784	112	1.2 (0.9 - 1.6)	0.272	61	0.9 (0.6 - 1.3)	0.528
		AA	34	40	1.9 (1.2 - 3.2)	**0.009**	6	2.6 (0.9 - 7.2)	0.07	15	1.5 (0.8 - 2.9)	0.227	17	2.2 (1.2 - 4.2)	0.016
FGF10 rs16901816 (T:G)	0.288														
Hispanic	0.380	TT	165	95	Reference		9	Reference		44	Reference		40	Reference	
		TG	214	94	0.9 (0.6 - 1.2)	0.433	14	1.1 (0.4 - 2.6)	0.860	38	0.8 (0.5 - 1.3)	0.368	39	0.9 (0.5 - 1.4)	0.541
		GG	60	26	0.9 (0.5 - 1.5)	0.593	1	NC		12	0.9 (0.4 - 1.9)	0.856	12	0.9 (0.4 - 1.9)	0.763
White	0.191	TT	164	175	Reference		30	Reference		105	Reference		36	Reference	
		TG	88	79	0.8 (0.6 - 1.2)	0.383	13	0.8 (0.4 - 1.8)	0.661	50	1.0 (0.6 - 1.5)	0.913	15	0.7 (0.3 - 1.3)	0.258
		GG	5	20	**4.1 (1.5 - 11.4)**	**0.007**	3	3.1 (0.6 - 14.9)	0.157	13	**5.2 (1.7 - 16.0)**	**0.004**	3	2.9 (0.6 - 14.1)	0.178
rs6892212 (A:C)	0.397														
Hispanic	0.463	AA	115	66	Reference		5	Reference		30	Reference		29	Reference	
		AC	236	115	1.0 (0.6 - 1.4)	0.799	18	1.6 (0.6 - 4.7)	0.353	47	0.9 (0.5 - 1.5)	0.669	47	0.9 (0.5 - 1.5)	0.634
		CC	83	33	0.8 (0.5 - 1.3)	0.334	1	NC		16	0.9 (0.5 - 1.8)	0.794	15	0.7 (0.4 - 1.5)	0.422
White	0.227	AA	147	162	Reference		28	Reference		99	Reference		31	Reference	
		AC	102	87	0.8 (0.5 - 1.1)	0.166	14	0.7 (0.3 - 1.5)	0.362	55	0.9 (0.6 - 1.4)	0.623	17	0.7 (0.3 - 1.3)	0.232
		CC	7	23	**3.3 (1.4 - 8.2)**	**0.008**	4	2.5 (0.7 - 9.7)	0.175	13	3.7 (1.3 - 10.1)	0.012	5	4.0 (1.1 - 14.4)	0.037

Gene, SNP (Alleles)	MAF (Con-trols)	Geno-type	No. Controls	No. Cases	OR (95% CI) All Cases	P	No. Mild Cases	OR (95% CI) Mild Cases	P	No. Moderate	OR (95% CI) Moderate Cases	P	No. Severe Cases	OR (95% CI) Severe Cases	P
rs2973644 (T:C)	0.331														
Hispanic	0.427	TT	138	80	Reference		7	Reference		37	Reference		34	Reference	
		TC	227	106	0.9 (0.6 - 1.3)	0.625	16	1.3 (0.5 - 3.4)	0.565	43	0.8 (0.5 - 1.4)	0.533	44	0.9 (0.5 - 1.4)	0.563
		CC	74	28	0.8 (0.4 - 1.3)	0.326	1	NC		14	0.9 (0.5 - 1.9)	0.833	12	0.7 (0.3 - 1.5)	0.354
White	0.200	TT	160	172	Reference		29	Reference		103	Reference		36	Reference	
		TC	93	78	0.8 (0.5 - 1.1)	0.182	13	0.7 (0.4 - 1.6)	0.447	51	0.9 (0.6 - 1.5)	0.728	13	0.5 (0.2 - 1.1)	0.073
		CC	5	21	**4.4 (1.6 - 12.1)**	**0.005**	4	4.1 (1.0 - 17.7)	0.058	12	**5.0 (1.6 - 15.6)**	**0.005**	4	3.6 (0.8 - 15.5)	0.084
rs1482679 (A:G)	0.453	AA	258	244	Reference		33	Reference		120	Reference		87	Reference	
		AG	399	253	0.8 (0.6 - 1.0)	0.029	34	0.9 (0.5 - 1.5)	0.681	135	0.8 (0.6 - 1.2)	0.311	76	0.6 (0.4 - 0.9)	0.005
		GG	180	124	0.9 (0.7 - 1.2)	0.469	14	1.0 (0.5 - 2.1)	0.979	62	1.0 (0.7 - 1.5)	0.976	45	0.8 (0.5 - 1.2)	0.217
rs2973646 (C:A)	0.326														
Hispanic	0.425														
		CC	138	80	Reference		7	Reference		37	Reference		34	Reference	
		AC	228	106	0.9 (0.6 - 1.3)	0.623	16	1.3 (0.5 - 3.4)	0.560	43	0.8 (0.5 - 1.4)	0.532	44	0.9 (0.5 - 1.4)	0.563
		AA	72	28	0.8 (0.5 - 1.3)	0.384	1	NC		14	1.0 (0.5 - 2.0)	0.895	12	0.7 (0.3 - 1.5)	0.401
White	0.198	CC	161	173	Reference		29	Reference		104	Reference		36	Reference	
		AC	92	78	0.8 (0.5 - 1.2)	0.241	13	0.8 (0.4 - 1.7)	0.548	51	1.0 (0.6 - 1.5)	0.850	13	0.5 (0.3 - 1.1)	0.082
		AA	5	22	**4.5 (1.6 - 12.4)**	**0.004**	4	4.2 (1.0 - 18.1)	0.055	13	**5.2 (1.7 - 16.0)**	**0.004**	4	3.6 (0.8 - 15.6)	0.082
GLI1 rs10783827 (T:G)	0.496	TT	229	193	Reference		33	Reference		93	Reference		59	Reference	
		TG	385	269	1.0 (0.8 - 1.3)	0.925	37	0.7 (0.4 - 1.2)	0.227	143	1.3 (0.9 - 1.8)	0.123	86	1.0 (0.6 - 1.4)	0.802
		GG	223	152	1.2 (0.9 - 1.6)	0.254	11	0.5 (0.2 - 1.1)	0.084	78	1.9 (1.3 - 2.9)	**0.002**	60	1.1 (0.7 - 1.7)	0.740
rs3825077 (G:A)	0.434														
Hispanic	0.355	GG	176	82	Reference		7	Reference		42	Reference		31	Reference	
		AG	212	98	1.0 (0.7 - 1.4)	0.846	14	1.3 (0.5 - 3.4)	0.584	37	0.7 (0.4 - 1.2)	0.217	43	1.2 (0.7 - 2.0)	0.447
		AA	49	29	1.1 (0.7 - 1.9)	0.657	3	1.4 (0.3 - 6.2)	0.623	10	0.7 (0.3 - 1.6)	0.417	16	1.7 (0.8 - 3.3)	0.162
White	0.372	GG	31	53	Reference		8	Reference		34	Reference		10	Reference	
		AG	126	142	0.7 (0.4 - 1.2)	0.159	24	0.7 (0.3 - 1.7)	0.429	84	0.6 (0.4 - 1.2)	0.162	32	0.8 (0.3 - 2.0)	0.670
		AA	96	77	**0.4 (0.2 - 0.7)**	**0.003**	13	0.6 (0.2 - 1.8)	0.397	49	**0.4 (0.2 - 0.8)**	**0.005**	12	**0.3 (0.1 - 0.8)**	**0.023**
rs3782126 (G:A)	0.471	GG	250	171	Reference		16	Reference		87	Reference		66	Reference	

Gene, SNP (Alleles)	MAF (Con-trols)	Geno-type	No. Controls	No. Cases	OR (95% CI) All Cases	P	No. Mild Cases	OR (95% CI) Mild Cases	P	No. Moderate	OR (95% CI) Moderate Cases	P	No. Severe Cases	OR (95% CI) Severe Cases	P
		AG	393	288	0.9 (0.7 - 1.2)	0.456	33	0.9 (0.4 - 1.7)	0.672	148	0.8 (0.6 - 1.1)	0.169	98	1.0 (0.7 - 1.4)	0.925
		AA	200	152	0.7 (0.5 - 1.0)	0.083	31	1.3 (0.7 - 2.8)	0.42	75	**0.5 (0.3 - 0.8)**	**0.002**	42	0.8 (0.5 - 1.3)	0.351
rs2292657 (C:T)	0.45	CC	268	184	Reference		16	Reference		97	Reference		69	Reference	
		TC	390	294	0.9 (0.7 - 1.2)	0.628	41	1.1 (0.6 - 2.2)	0.675	152	0.8 (0.6 - 1.1)	0.246	93	0.9 (0.6 - 1.4)	0.747
		TT	183	140	0.8 (0.5 - 1.0)	0.086	23	1.2 (0.6 - 2.5)	0.641	69	**0.5 (0.3 - 0.8)**	**0.004**	43	0.8 (0.5 - 1.3)	0.333
rs4760259 (C:T)	0.49	CC	242	199	Reference		32	Reference		104	Reference		55	Reference	
		TC	355	264	1.1 (0.8 - 1.4)	0.568	38	0.9 (0.5 - 1.5)	0.621	131	1.2 (0.9 - 1.7)	0.206	90	1.2 (0.8 - 1.7)	0.460
		TT	228	151	1.2 (0.9 - 1.7)	0.244	11	0.5 (0.2 - 1.2)	0.123	78	**1.8 (1.2 - 2.8)**	**0.004**	60	1.2 (0.7 - 1.9)	0.498
rs2228226 (C:G)	0.469	CC	256	203	Reference		33	Reference		103	Reference		59	Reference	
		GC	379	284	1.1 (0.9 - 1.4)	0.392	38	0.8 (0.5 - 1.4)	0.484	145	1.4 (1.0 - 1.9)	0.059	96	1.1 (0.8 - 1.7)	0.499
		GG	205	132	1.2 (0.9 - 1.6)	0.282	10	0.6 (0.3 - 1.3)	0.178	67	1.8 (1.1 - 2.7)	**0.009**	53	1.1 (0.7 - 1.8)	0.577
GLI2 rs4848125 (A:G)	0.356														
Hispanic	0.355	AA	173	96	Reference		8	Reference		45	Reference		42	Reference	
		AG	192	84	0.8 (0.6 - 1.1)	0.221	10	1.1 (0.4 - 3.0)	0.808	35	0.7 (0.4 - 1.1)	0.135	34	0.8 (0.5 - 1.2)	0.271
		GG	52	28	0.9 (0.5 - 1.6)	0.748	4	1.9 (0.5 - 6.7)	0.349	11	0.7 (0.3 - 1.6)	0.417	13	1.0 (0.5 - 2.0)	0.915
White	0.402	AA	85	73	Reference		17	Reference		38	Reference		17	Reference	
		AG	117	117	1.2 (0.8 - 1.8)	0.516	15	0.6 (0.3 - 1.4)	0.23	77	1.5 (0.9 - 2.6)	0.109	22	1.0 (0.5 - 2.0)	0.952
		GG	38	68	**2.3 (1.4 - 3.9)**	**0.002**	13	1.5 (0.6 - 3.5)	0.361	40	**2.8 (1.5 - 5.4)**	**0.001**	13	2.1 (0.9 - 5.0)	0.104
rs4143116 (G:A)	0.317	GG	389	293	Reference		44	Reference		150	Reference		89	Reference	
		AG	345	236	1.0 (0.8 - 1.3)	0.833	27	0.8 (0.5 - 1.4)	0.456	118	1.1 (0.8 - 1.5)	0.466	87	1.1 (0.7 - 1.5)	0.740
		AA	89	82	1.4 (1.0 - 2.1)	0.056	7	1.1 (0.5 - 2.9)	0.765	42	**2.1 (1.3 - 3.3)**	**0.004**	32	1.1 (0.6 - 1.9)	0.725
rs4848126 (G:A)	0.413														
Hispanic	0.399	GG	156	91	Reference		9	Reference		40	Reference		41	Reference	
		AG	210	89	0.7 (0.5 - 1.0)	0.074	10	0.8 (0.3 - 2.2)	0.732	40	0.7 (0.4 - 1.2)	0.159	34	0.6 (0.4 - 1.0)	0.072
		AA	68	34	0.8 (0.5 - 1.3)	0.309	5	1.7 (0.5 - 5.6)	0.385	13	0.6 (0.3 - 1.1)	0.110	16	0.8 (0.4 - 1.6)	0.569
White	0.428	GG	86	68	Reference		15	Reference		39	Reference		13	Reference	
		AG	122	131	1.4 (0.9 - 2.1)	0.11	16	0.7 (0.3 - 1.5)	0.369	86	1.7 (1.0 - 2.7)	0.047	27	1.7 (0.8 - 3.6)	0.162
		AA	49	73	**2.0 (1.2 - 3.3)**	**0.007**	14	1.4 (0.6 - 3.2)	0.483	42	2.1 (1.2 - 3.8)	0.015	14	2.2 (0.9 - 5.3)	0.071
GLI3 rs6974655 (G:T)	0.234	GG	498	361	Reference		39	Reference		184	Reference		126	Reference	

Gene, SNP (Alleles)	MAF (Con-trols)	Geno-type	No. Controls	No. Cases	OR (95% CI) All Cases	P	No. Mild Cases	OR (95% CI) Mild Cases	P	No. Moderate	OR (95% CI) Moderate Cases	P	No. Severe Cases	OR (95% CI) Severe Cases	P
		TG	295	212	0.9 (0.7 - 1.2)	0.619	29	1.1 (0.6 - 1.8)	0.775	115	1.0 (0.8 - 1.4)	0.871	66	0.9 (0.6 - 1.3)	0.595
		TT	50	47	1.2 (0.8 - 1.8)	0.475	12	**3.3 (1.5 - 7.6)**	**0.004**	18	0.7 (0.4 - 1.3)	0.294	16	1.2 (0.6 - 2.2)	0.595
rs9886211 (A:G)	0.221														
Hispanic	0.205	AA	280	148	Reference		12	Reference		70	Reference		64	Reference	
		AG	138	59	0.8 (0.6 - 1.2)	0.275	11	2.0 (0.8 - 5.0)	0.112	19	0.5 (0.3 - 0.9)	0.02	25	0.8 (0.5 - 1.3)	0.389
		GG	21	7	0.7 (0.3 - 1.6)	0.387	1	0.9 (0.1 - 7.6)	0.917	4	0.8 (0.3 - 2.5)	0.726	2	0.5 (0.1 - 2.1)	0.326
White	0.282	AA	139	130	Reference		24	Reference		85	Reference		19	Reference	
		AG	91	120	1.5 (1.0 - 2.2)	0.035	17	1.1 (0.5 - 2.2)	0.837	72	1.4 (0.9 - 2.2)	0.105	27	**2.4 (1.2 - 4.8)**	**0.009**
		GG	27	22	0.9 (0.5 - 1.7)	0.815	4	0.9 (0.3 - 3.0)	0.883	10	0.7 (0.3 - 1.5)	0.313	8	2.4 (0.9 - 6.3)	0.081
rs3801223 (C:T)	0.447	CC	273	189	Reference		29	Reference		98	Reference		58	Reference	
		TC	381	280	1.2 (0.9 - 1.5)	0.196	38	0.9 (0.5 - 1.6)	0.734	137	1.2 (0.9 - 1.7)	0.226	97	1.2 (0.8 - 1.8)	0.261
		TT	183	152	1.3 (1.0 - 1.8)	0.056	14	0.7 (0.3 - 1.4)	0.279	82	1.7 (1.2 - 2.5)	**0.007**	53	1.2 (0.8 - 2.0)	0.329
SHH rs9333613 (A:G)	0.026	AA	787	574	Reference		79	Reference		291	Reference		189	Reference	
		AG	37	37	1.4 (0.8 - 2.4)	0.209	1	NC		20	**2.4 (1.2 - 4.7)**	**0.009**	16	1.4 (0.7 - 2.7)	0.385
		GG	3	1	NC		0	NC		0	NC		1	NC	
WT1 rs1799937 (T:C)	0.387	TT	323	242	Reference		31	Reference		140	Reference		63	Reference	
		TC	388	267	1.1 (0.8 - 1.3)	0.654	43	1.6 (0.9 - 2.7)	0.079	131	0.9 (0.7 - 1.2)	0.570	87	1.1 (0.8 - 1.6)	0.547
		CC	133	113	1.4 (1.0 - 1.9)	0.077	7	1.2 (0.5 - 2.9)	0.76	46	1.1 (0.7 - 1.7)	0.739	59	**1.9 (1.2 - 3.0)**	**0.006**
rs5030277 (T:A)	0.268	TT	462	363	Reference		46	Reference		210	Reference		98	Reference	
		TA	296	185	1.0 (0.8 - 1.3)	0.856	28	1.5 (0.8 - 2.5)	0.189	83	0.9 (0.6 - 1.2)	0.520	70	1.1 (0.7 - 1.6)	0.758
		AA	75	64	1.5 (1.0 - 2.3)	0.035	4	1.1 (0.4 - 3.6)	0.842	20	1.1 (0.6 - 1.9)	0.845	40	**2.3 (1.4 - 3.9)**	**0.001**
rs16754 (A:G)	0.289	AA	440	332	Reference		42	Reference		198	Reference		83	Reference	
		AG	310	217	1.2 (0.9 - 1.5)	0.215	34	1.8 (1.1 - 3.2)	0.026	94	0.9 (0.7 - 1.3)	0.596	83	1.4 (0.9 - 2.0)	0.098
		GG	88	72	1.5 (1.0 - 2.3)	0.03	5	1.3 (0.5 - 3.8)	0.619	25	1.1 (0.7 - 2.0)	0.642	42	**2.3 (1.4 - 3.9)**	**0.001**
rs5030234 (C:A)	0.286	CC	436	342	Reference		44	Reference		196	Reference		93	Reference	
		AC	320	209	1.0 (0.8 - 1.3)	0.866	32	1.4 (0.8 - 2.4)	0.18	96	0.9 (0.7 - 1.2)	0.504	75	1.0 (0.7 - 1.5)	0.912
		AA	79	70	1.5 (1.0 - 2.3)	0.034	5	1.2 (0.4 - 3.5)	0.7	24	1.1 (0.7 - 2.0)	0.632	41	**2.1 (1.3 - 3.5)**	**0.003**
rs12293750 (C:A)	0.026	CC	796	580	Reference		71	Reference		300	Reference		195	Reference	
		AC	36	40	1.5 (0.9 - 2.4)	0.126	10	**3.9 (1.6 - 9.3)**	**0.002**	16	0.9 (0.5 - 1.8)	0.794	13	1.5 (0.8 - 3.0)	0.252

Gene, SNP (Alleles)	MAF (Con-trols)	Geno-type	No. Controls	No. Cases	OR (95% CI) All Cases	P	No. Mild Cases	OR (95% CI) Mild Cases	P	No. Moderate	OR (95% CI) Moderate Cases	P	No. Severe Cases	OR (95% CI) Severe Cases	P
		AA	4	1	NC		0	NC		0	NC		1	NC	
rs3858449 (G:A)	0.309	GG	414	324	Reference		45	Reference		185	Reference		86	Reference	
		AG	325	213	1.0 (0.8 - 1.3)	0.939	27	1.2 (0.7 - 2.1)	0.524	103	0.9 (0.7 - 1.3)	0.608	76	1.1 (0.7 - 1.5)	0.765
		AA	96	79	1.4 (1.0 - 2.1)	0.063	8	2.0 (0.8 - 4.9)	0.146	26	1.0 (0.6 - 1.6)	0.862	45	**2.0 (1.2 - 3.2)**	**0.007**

*Results for SNPs with p-value <0.01 overall or within a specific phenotype are shown (ORs with p<0.01 are in **bold**). ORs are presented if all cells in the comparison had at least 3 observations; separate results for whites and Hispanics are shown if the p-value for interaction was <0.10. All odds ratios were adjusted for the two ancestral proportion variables, maternal residence in the Central Valley (yes/no), and maternal race-ethnicity (Hispanic, non-Hispanic white, or other) if the results were not already stratified.

MAF = minor allele frequency, NC = not calculated

Table 4

Association of risk scores overall and within specific phenotypes.*

	Score	No. Cases	No. Controls	OR (95% CI)
All Cases	0	453	726	Reference
	1	144	106	2.0 (1.5, 2.7)
	2	23	12	2.5 (1.2, 5.4)
	3	4	0	undefined
Mild Cases	0	61	764	Reference
	1	18	74	3.5 (1.8, 7.0)
	2	2	6	6.0 (0.9, 41.8)
Moderate Cases	0	104	339	Reference
	1	130	367	1.5 (1.1, 2.2)
	2	59	112	2.8 (1.8, 4.3)
	3	22	24	8.1 (3.8, 17.0)
	4	4	2	32.6 (5.0, 211.4)
Severe Cases	0	66	404	Reference
	1	93	352	1.6 (1.1, 2.3)
	2	47	82	3.4 (2.1, 5.6)
	3	3	6	3.7 (0.8, 16.2)

* Risk scores reflect the number of genes for which an individual had a variant genotype that had a p-value <0.01 (see Table 3 for variants that met this criterion). The maximum possible scores were 4, 2, 6, and 4 for all, mild, moderate and severe cases, respectively. All odds ratios were adjusted for the two ancestral proportion variables, maternal residence in the Central Valley (yes/no), and maternal race-ethnicity (Hispanic, non-Hispanic white, or other) if the results were not already stratified.