Phenotype Specific Association of the *TGFBR3* Locus with Nonsyndromic Cryptorchidism

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Purpose: Based on a genome-wide association study of testicular dysgenesis syndrome showing a possible association with *TGFBR3*, we analyzed data from a larger, phenotypically restricted cryptorchidism population for potential replication of this signal.

Materials and Methods: We excluded samples based on strict quality control criteria, leaving 844 cases and 2,718 controls of European ancestry that were analyzed in 2 separate groups based on genotyping platform (ie Illumina® HumanHap550, version 1 or 3, or Human610-Quad, version 1 BeadChip in group 1 and Human OmniExpress 12, version 1 BeadChip platform in group 2). Analyses included genotype imputation at the *TGFBR3* locus, association analysis of imputed data with correction for population substructure, subsequent meta-analysis of data for groups 1 and 2, and selective genotyping of independent cases (330) and controls (324) for replication. We also measured *Tgfbr3* mRNA levels and performed TGFBR3/betaglycan immunostaining in rat fetal gubernaculum.

Results: We identified suggestive ($p \leq 1\times10^{-4}$) association of markers in/near *TGFBR3*, including rs9661103 (OR 1.40; 95% CI 1.20, 1.64; $p = 2.71\times10^{-5}$) and rs10782968 (OR 1.58; 95% CI 1.26, 1.98; $p = 9.36\times10^{-5}$) in groups 1 and 2, respectively. In subgroup analyses we observed strongest association of rs17576372 (OR 1.42; 95% CI 1.24, 1.60; $p = 1.67\times10^{-4}$) with proximal and rs11165059 (OR 1.32; 95% CI 1.15, 1.38; $p = 9.42\times10^{-4}$) with distal testis position, signals in strong linkage disequilibrium with rs9661103 and rs10782968, respectively. Association of the prior genome-wide association study signal (rs12082710) was marginal (OR 1.13; 95% CI 0.99, 1.28; $p = 0.09$ for group 1), and we were unable to replicate signals in our independent cohort. *Tgfbr3*/betaglycan was differentially expressed in wild-type and cryptorchid rat fetal gubernaculum.

Conclusions: These data suggest complex or phenotype specific association of cryptorchidism with *TGFBR3* and the gubernaculum as a potential target of TGFβ signaling.

Abbreviations and Acronyms

DHT = dihydrotestosterone

E = embryonic day

GWAS = genome-wide association study

INSL3 = insulin-like 3

LD = linkage disequilibrium

RAF = risk allele frequency

SNP = single nucleotide polymorphism

TDS = testicular dysgenesis syndrome

TGFBR3 = transforming growth factor-β receptor 3

wt = wild-type

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CRYPTORCHIDISM is a common genital anomaly identified in boys at birth or during childhood. The etiology is poorly understood and likely multifactorial, although associated genetic loci remain largely unknown. Familial aggregation suggests moderate genetic susceptibility and implicates the maternal environment as contributory to cryptorchidism risk.[1,2] Level of familial risk in this disorder exceeds that for many complex diseases and predicts the potential for greater success in genome-wide analyses.[3]

Testicular descent is regulated by insulin-like 3 and androgens, Leydig cell derived hormones that control development of the fetal gubernaculum.[4] Exonic variants of *INSL3*, its receptor relaxin/insulin-like family peptide receptor 2 (*RXFP2*) or other hormone pathway genes are rare in cryptorchidism cases, and their functional significance is poorly defined.[5] Dalgaard et al performed a GWAS augmented by systems biology analysis methodology to identify genetic markers linked to the testicular dysgenesis syndrome, which includes cryptorchidism, hypospadias, testicular cancer and infertility.[6] This approach did not identify genome-wide significant signals, but had limited power to detect loci associated with isolated cryptorchidism. However, a *TGFBR3* intronic single nucleotide polymorphism, rs12082710, demonstrated evidence suggestive of association with TDS and cryptorchidism. Accordingly we focused on *TGFBR3* in an initial analysis of data from a larger GWAS cohort, and observed suggestive, phenotype-specific association of nonsyndromic cryptorchidism with this locus.

MATERIAL AND METHODS

Subjects and Genotyping

Subjects included boys with cryptorchidism who underwent surgical repair at Nemours/Alfred I. duPont Hospital for Children or the Children's Hospital of Philadelphia. Exclusion criteria consisted of multiple congenital anomalies and/or diagnosis of a syndrome, other genital anomalies (hypospadias, chordee or other penile anomalies) and abdominal wall defects or major urogenital malformations. Control subjects recruited through the Children's Hospital of Philadelphia Care Network were males 7 years or older without a history of testicular disease, syndromes or other medical disorders potentially associated with cryptorchidism, including inguinal hernia and hypospadias.

Basic demographic and phenotypic data were collected, including age at diagnosis, race, ethnicity, laterality and position of the affected testis. Blood sample or excess tissue was collected and stored at −80C or in RNAlater® solution. As described previously,[7] we categorized the cases into different phenotypic subgroups. Nonscrotal position was defined as distal if the most severely affected cryptorchid testis was located at or beyond the external inguinal ring and proximal if at least 1 testis was located within the inguinal canal or abdomen. We assigned boys 2 years or younger to the early subgroup and those older than 2 years to the late subgroup based on timing of surgery by a pediatric urologist. Informed consent was obtained for all participants based on approval of the institutional review board at each participating center.

DNA was extracted from tissue or blood samples (5 PRIME, Inc., Gaithersburg, Maryland), and whole genome amplification (REPLI-g Mini Kit, Qiagen, Germantown, Maryland) was performed for those with low DNA yield. Samples of adequate purity (optical density 260/280 ratio of 1.8 to 2.0 by NanoDrop® 1000 spectrophotometer) were entered into the standard genotyping work flow at the Center for Applied Genomics at the Children's Hospital of Philadelphia. Two separate groups were analyzed based on the genotyping platform used in the discovery stage, according to availability of control genotypes. Group 1 consisted of 559 cases and 1,772 controls that were genotyped using HumanHap550, version 1 or 3, or Human610-Quad, version 1 BeadChip (Illumina). These platforms have more than 535,000 SNPs in common. Group 2 consisted of 353 cases and 1,149 controls that were genotyped using the Human OmniExpress 12, version 1 BeadChip platform (Illumina).

Discovery Phase Data Analysis

Genome-wide genotyping data from groups 1 and 2 were analyzed separately using PLINK, version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/).[8,9] SNP content differed slightly for each of the 3 genotyping platforms used in group 1. Therefore, only overlapping SNPs (535,752) were used for subsequent analysis. Individuals were excluded from further analysis due to 1) discordance between reported sex and X and Y chromosome SNP data, 2) missing genotype rate greater than 3%, 3) higher or lower than expected heterozygosity rate (greater than 3 SDs from mean) and 4) duplicates or relatives (based on estimate of proportion of alleles shared identical by descent greater than 0.1875). SNPs were excluded due to 1) missing genotype rate greater than 5%, 2) Hardy-Weinberg equilibrium deviation in controls ($p < 0.00001$), 3) significantly different missing genotype rates between cases and controls ($p < 0.00001$), and 4) low minor allele frequency (less than 0.01). To select samples of European ancestry and control for population substructure, multidimensional scaling analysis was performed in PLINK using European population SNP genotyping data from the Stanford Human Genome Diversity Project (http://www.hagsc.org/hgdp/files.html).[10] We removed all samples that deviated from the means of the first or second components by more than 3 SDs. We performed separate association analyses for the remaining samples in groups