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

1 and 2 using logistic regression with multidimensional scaling components 1 and 2 as covariates.

We used HaploView, version 4.2 to define the pattern of LD at the *TGFBR3* locus (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview),[11] and a targeted region was chosen for imputation (Chr1:91960000-92415000, GRCh37/hg19 assembly). Genotype data in PLINK format were converted to IMPUTE, version 2.3.0 file format,[12,13] using GTOOL, version 0.7.5 (http://www.well.ox.ac.uk/~cfreeman/software/gwas/gtool.html). We performed imputation using the 1000 Genomes Project reference population (September 2013 version), followed by association analysis of imputed data using IMPUTE2 and SNPTEST, version 2.5β (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/old/snptest_v2.3.0.html), respectively.[12,13] Imputed SNPs were removed from SNPTEST results based on the criteria minor allele frequency less than 0.01, control Hardy-Weinberg equilibrium less than 0.00001 and frequentist_add_info (imputation quality score) less than 0.8. Meta-analyses of the remaining SNPs for groups 1 and 2 was performed using META, version 1.5 (http://www.stats.ox.ac.uk/~jsliu/meta.html) for all cases and controls, and for subgroups defined by testicular position, laterality and timing of presentation. Differences in phenotype frequencies between the 2 discovery groups were analyzed using chi-square tests. Results were mapped using the SNAP Web tool (http://www.broadinstitute.org/mpg/snap/),[14] and LD among the strongest signals was estimated using HaploView.

Replication Phase

DNA samples from boys with cryptorchidism and unaffected controls were obtained in a prior study of cryptorchidism in Sweden.[15–17] We selected the top *TGFBR3* signals from groups 1 and 2, or an SNP in complete LD, and the previously published marker rs12082710 for genotyping using TaqMan® assays.[6] Genotyping was performed in a 384-well format using the Applied Biosystems® PRISM® 7900HT fast real-time polymerase chain reaction system. A reaction volume of 5 μl included 1.25 μl water, 2.5 μl 2 × Applied Biosystems genotyping master mix, 0.25 μl 20 × TaqMan genotyping assay and 1 μl (15 ng) genomic DNA. The polymerase chain reaction conditions were 95C for 10 minutes, followed by 50 cycles at 95C for 15 seconds and 60C for 60 seconds. The genotyping calls were made by Applied Biosystems SDS Automation Controller software, version 2.3. Association tests were performed using online software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). A description of supplementary methods used to conduct real-time quantitative polymerase chain reaction and immunostaining is available online (http://jurology.com/).[18–20]

RESULTS

Based on quality control criteria, 56 of 559 cases, 158 of 1,772 controls and 28,749 of 535,752 markers were removed from group 1, and 12 of 353 cases, 45 of 1,149 controls and 89,489 of 719,629 markers were removed from group 2, leaving 844 cases and 2,718 controls in the final analysis. Q-Q plots of the distribution of p values in groups 1 and 2 did not exhibit significant deviation from their expected distribution, with genomic inflation factor (λ) values of 1.014 and 1.017, respectively.

There were no genome-wide significant ($p < 5.0\times10^{-8}$) signals in analyses of the 2 subsets in the discovery phase (groups 1 and 2), but we identified *TGFBR3* (chr1:92145900-92371559, reverse strand, GRCh37/hg19 assembly) as the only genomic region containing suggestive ($p \leq 1\times10^{-4}$) markers for both groups. In light of the previous data suggesting a possible association of *TGFBR3* with TDS and cryptorchidism,[6] we focused on this region initially. Following imputation the strongest signals were rs9661103 ($p = 2.71\times10^{-5}$), located in intron 1, and rs10782968 ($p = 9.38\times10^{-5}$), located approximately 101 kb downstream between *TGFBR3* and *CDC7*, for groups 1 and 2, respectively (fig. 1, table 1). However, the reciprocal analysis revealed no evidence of association of 3′ downstream or 5′ intragenic SNPs with groups 1 and 2, respectively, so these signals were weaker when we combined discovery data for groups 1 and 2 in a meta-analysis. In addition, we observed marginal (OR 1.13, 95% CI 0.99−1.28 $p = 0.09$) association of rs12082710 with group 1 but not group 2 cases. However, the RAF in our control samples (0.62) was higher than previously reported (0.58 to 0.59) for this variant,[6] and, therefore, was similar to the RAF in our case groups. In the replication phase available genotypes of 305 cases and 324 unaffected controls collected from white subjects in Sweden failed to show association of any of these markers with cryptorchidism (table 1).

We performed additional analyses to determine whether phenotypic heterogeneity between our 2 case groups could potentially account for these variable results. Testicular position, laterality and/or age at presentation data were available for at least 90% of affected individuals in each classification (table 2). We noted increased frequencies of proximal, bilateral and early presentation in group 1 as compared to group 2, although these trends were not statistically significant. However, in meta-analyses of subphenotypes we noted strongest evidence of association of proximal and distal testicular positioning with upstream/promoter and downstream regions of *TGFBR3*, respectively (fig. 2, supplementary table [http://jurology.com/]). For the additional meta-analyses based on laterality and age at presentation all associations were weaker.

Using HaploView, we reviewed the LD patterns for the top signals in the 2 genotyping groups and the position subgroups. The strongest signals in group 1 (rs9661103) and group 2 (rs10782968) analyses are not in LD with each other, but were

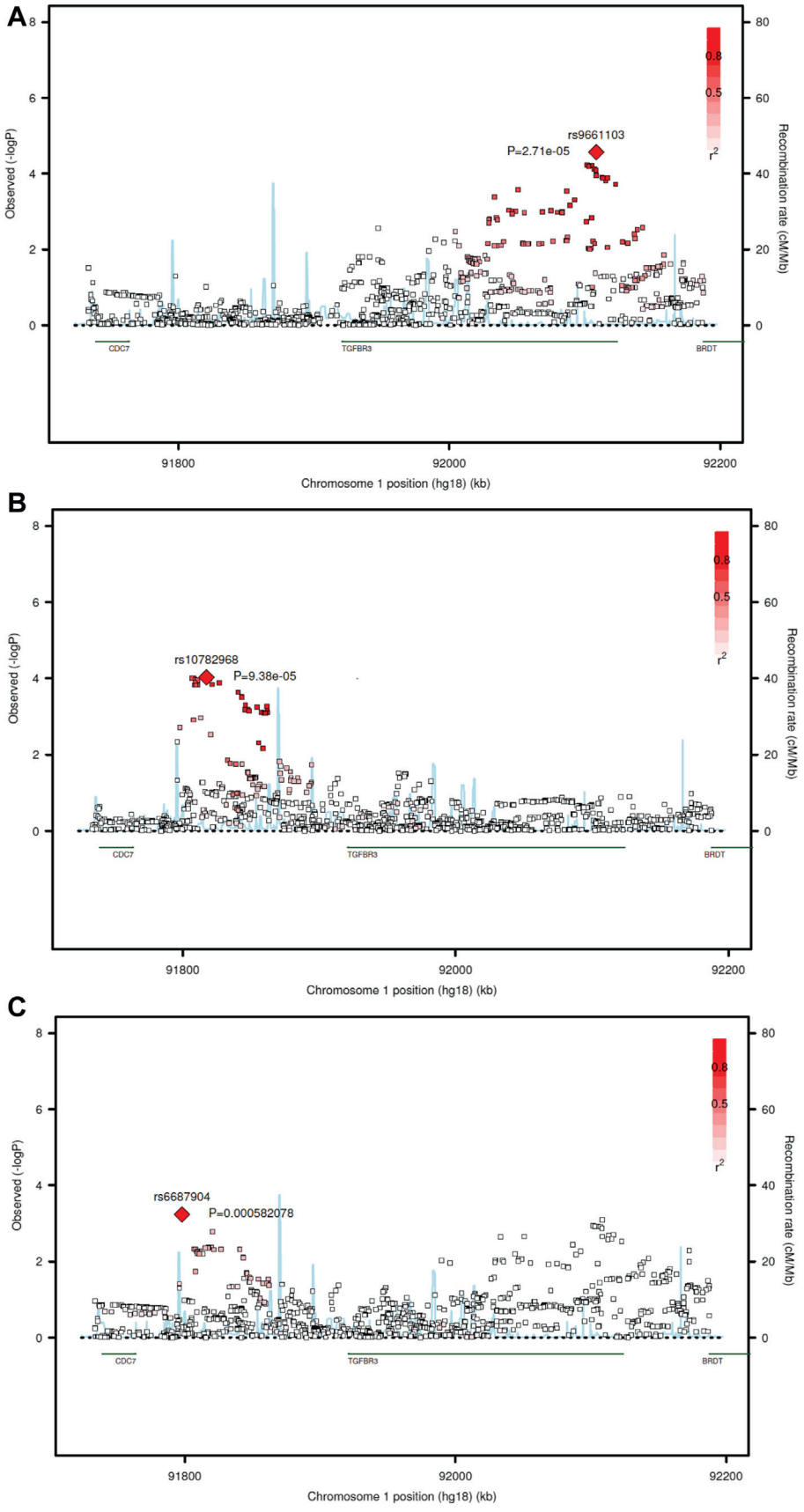

Figure 1. SNAP plots demonstrate association analysis results of imputed data. *A*, group 1 cases and controls. *B*, group 2 cases and controls. *C*, meta-analysis of all cases and controls.

Table 1. *Results of single marker association analysis in discovery and replication groups*

	RAF Cases	RAF Controls	OR (95% CI)	p Value
	Discovery group 1			
Marker rs6687904, position 92025262, risk allele G	0.89	0.87	1.20 (0.96, 1.50)	5.03E-02
Marker rs925192, position 92038225, risk allele T	0.83	0.82	1.01 (0.84, 1.22)	0.62
Marker rs10782968, position 92044755, risk allele T	0.79	0.78	1.02 (0.86, 1.21)	0.62
Marker rs12082710, position 92155337, risk allele T	0.65	0.62	1.13 (0.99, 1.28)	0.09
Marker rs9661103, position 92335906, risk allele C	0.74	0.67	**1.40 (1.20, 1.64)**	2.71E-05
	Discovery group 2			
Marker rs6687904, position 92025262, risk allele G	0.90	0.85	1.50 (1.40, 1.98)	1.91E-03
Marker rs925192, position 92038225, risk allele T	0.87	0.80	1.66 (1.29, 2.13)	1.12E-04
Marker rs10782968, position 92044755, risk allele T	0.84	0.76	**1.58 (1.26, 1.98)**	9.38E-05
Marker rs12082710, position 92155337, risk allele T	0.62	0.62	1.01 (0.85, 1.20)	0.74
Marker rs9661103, position 92335906, risk allele C	0.70	0.69	1.01 (0.84, 1.22)	0.88
	Discovery meta-analysis			
Marker rs6687904, position 92025262, risk allele G			**1.35 (1.14, 1.60)**	5.82E-04
Marker rs925192, position 92038225, risk allele T			1.23 (1.07, 1.43)	5.29E-03
Marker rs10782968, position 92044755, risk allele T			1.22 (1.06, 1.39)	4.22E-03
Marker rs12082710, position 92155337, risk allele T			1.07 (0.95, 1.18)	0.27
Marker rs9661103, position 92335906, risk allele C			1.23 (1.09, 1.38)	7.94E-04
	Replications			
Marker rs6687904, position 92025262, risk allele G				
Marker rs925192, position 92038225, risk allele T	0.82	0.81	1.07 (0.80, 1.43)	0.63
Marker rs10782968, position 92044755, risk allele T				
Marker rs12082710, position 92155337, risk allele T	0.62	0.61	1.04 (0.83, 1.30)	0.83
Marker rs9661103, position 92335906, risk allele C	0.65	0.69	0.84 (0.66, 1.06)	0.14

Markers shown include top signals (in bold) identified in groups 1 (rs9661103) and 2 (rs10782968 and rs925192) in present study, meta-analysis (rs6687904) and series by Dalgaard et al (rs12082710).[6]

correspondingly strongly linked ($r^2 \geq 0.8$) to the most significant SNPs that we identified in the proximal (rs17576372) and distal (rs6687904) meta-analyses (figs. 1 to 3), consistent with a greater frequency of proximal cases in group 1. These results suggest that phenotypic bias may be 1 factor accounting for the nonoverlapping signals we identified in separate analyses of our discovery groups.

In studies of rat gubernaculum *Tgfbr3* mRNA levels did not change significantly in wt samples between E17 and E21 ($p = 0.57$), the maximal developmental period (fig. 4). In contrast, *Tgfbr3* expression increased after E17 ($p = 0.055$) and was significantly greater in cryptorchid orl at E19 ($p = 0.011$) and E21 ($p = 0.001$) compared to wt gubernaculum. In wt gubernacular explants INSL3 exposure did not alter measured *Tgfbr3* expression, but transcript levels increased significantly following DHT exposure ($p < 0.01$ for 10 nM DHT).

Table 2. *Frequency of specific phenotypes by group*

Phenotype	Group 1	Group 2	p Value
No. position (%):			0.47
Proximal	167 (35)	107 (32)	
Distal	313 (65)	224 (68)	
No. laterality (%):			0.21
Bilat	137 (28)	80 (24)	
Unilat	360 (72)	258 (76)	
No. presentation (%):			0.07
Early	236 (48)	142 (42)	
Late	255 (52)	198 (58)	

Immunostaining of the rat fetal gubernaculum demonstrated diffuse TGFBR3 protein expression within the inner mesenchymal core but more intense staining of the developing peripheral muscle and outer mesothelial layer, as noted in E17 and E19 wt gubernacula (fig. 5, *A*). The muscle layer was adjacent to the mesothelial layer at E17 and became progressively less peripheral and thicker during development. Prominent clusters of betaglycan (TGFBR3-A positive) immunoreactivity consistent with the proteolytically cleaved extracellular domain (soluble betaglycan) revealed variable expression at all time points, along with coexpression of membrane associated betaglycan and myosin in differentiated muscle. These extracellular collections were not identified using TGFBR3-B, an antibody targeting cytoplasmic betaglycan, and expression was less prominent by E21 in wt and orl samples. These collections were particularly prominent in some orl samples, particularly within the mesenchymal core and the peripheral mesothelial layer (fig. 5, *B*).

DISCUSSION

Cryptorchidism is the most common reproductive anomaly identified in newborn boys but genetic susceptibility loci remain largely undefined. In the sole published GWAS that includes cryptorchidism *TGFBR3* was identified as a locus associated with TDS and less strongly with isolated nonsyndromic cryptorchidism, although the data did not approach

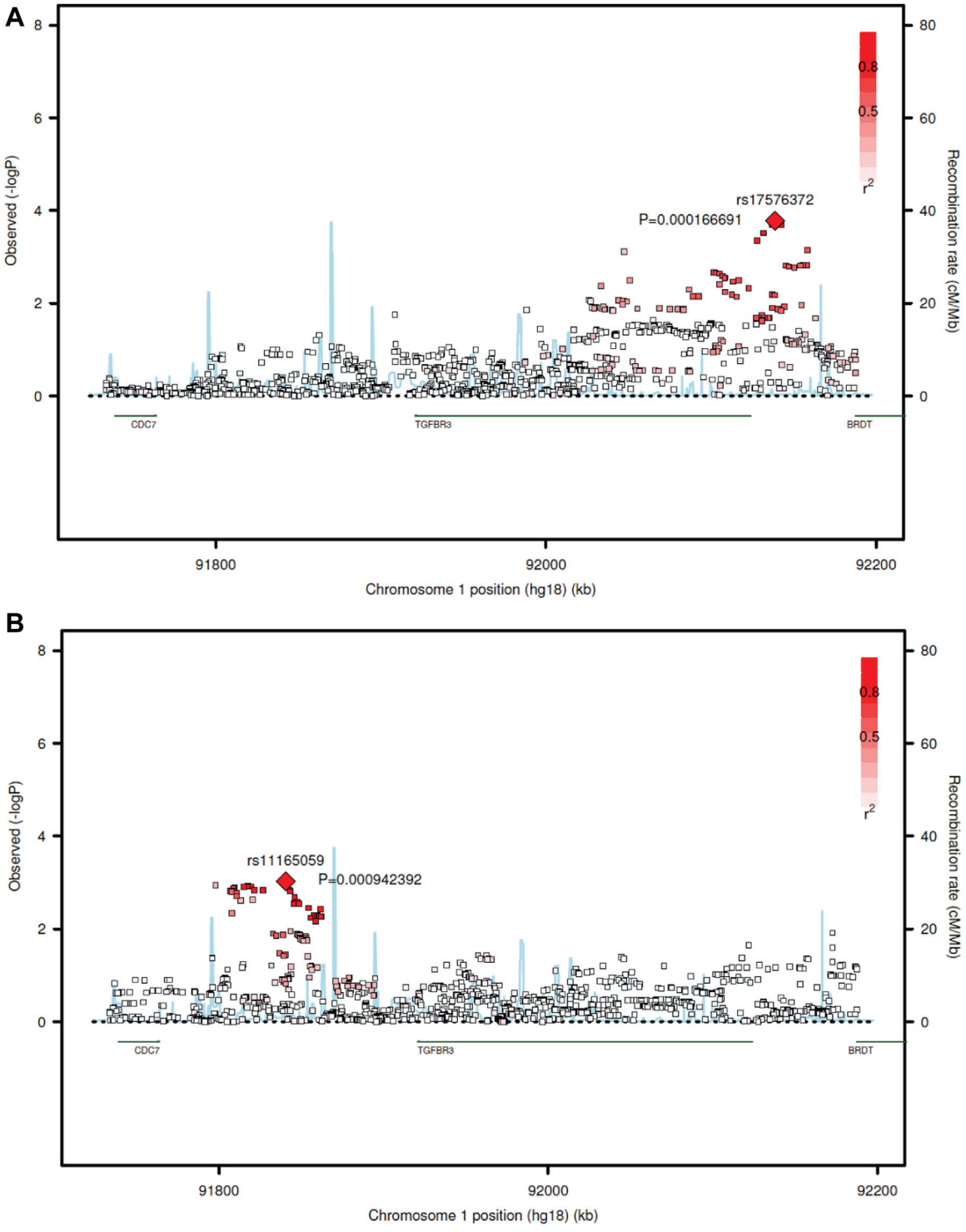

Figure 2. SNAP plots of meta-analysis results of imputed data at *TGFBR3* locus. *A*, cryptorchid cases with testes located proximal to external inguinal ring and all controls. *B*, cryptorchid cases with testes located distal to external inguinal ring.

genome-wide significance.[6] In the present analysis the larger sample size provided increased power to detect an association of *TGFBR3* with cryptorchidism. Although we showed nominal association of our group 1 cohort with the previously reported signal, this association was lost with increasing sample size and was not replicated in a Swedish population. Surprisingly we observed suggestive ($p < 10^{-4}$) association of each of our case-control groups with independent signals at or near the

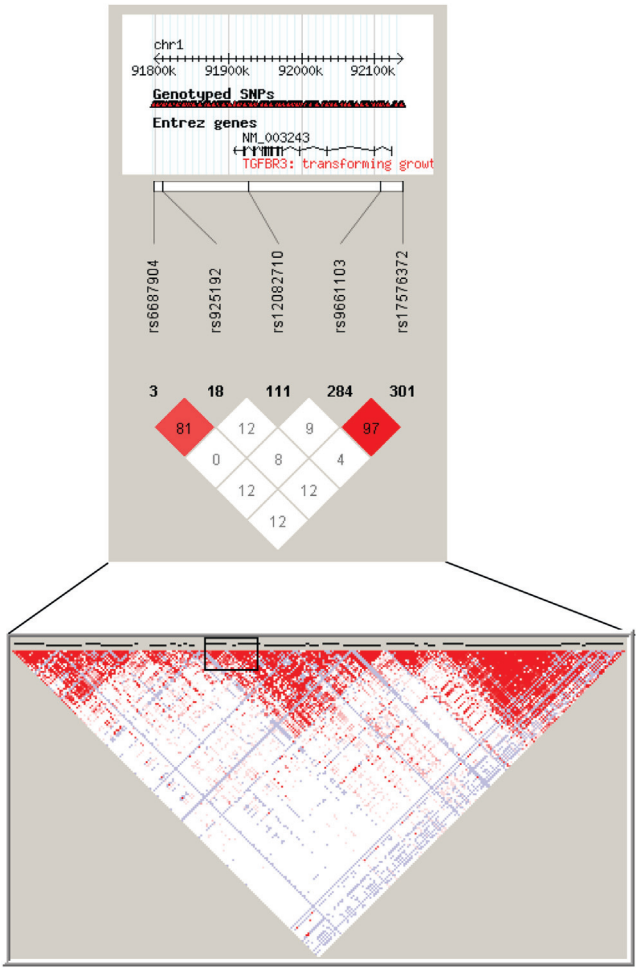

Figure 3. LD plot of markers identified in primary group and subgroup analyses (available in HaploView). Lower portion of figure illustrates overview of LD blocks within imputed *TGFBR3* locus. Black rectangle is centered on 3′ end of *TGFBR3* gene.

TGFBR3 locus. Our subphenotype analyses suggest stronger association of proximal and distal phenotypes with linked SNPs in the 5′ and downstream

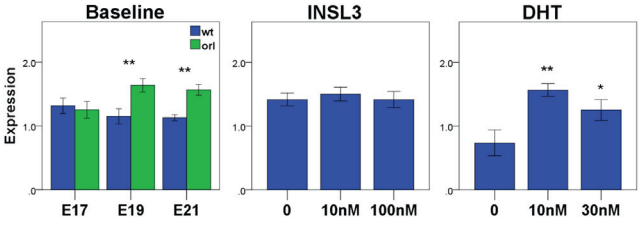

Figure 4. *Tgfbr3* mRNA expression in gubernaculum from wt and cryptorchid orl rat fetuses. Baseline expression is illustrated for freshly isolated E17, E19 and E21 gubernacula and for E17 wt gubernacular explants established in culture followed by exposure to INSL3 or DHT for 24 hours. Asterisk indicates $p < 0.05$ for wt vs orl. Double asterisk signifies $p < 0.01$ for wt vs orl.

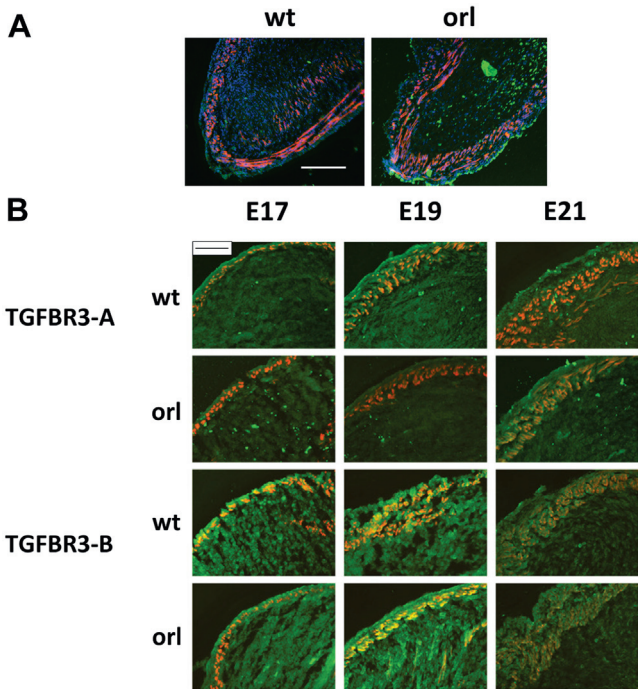

Figure 5. *A*, betaglycan expression (green stain) in E17, E19 and E21 gubernacula from wt and cryptorchid orl fetuses. Immunostaining reveals myosin (differentiated muscle, red) and TGFBR3-A (extracellular domain, green, top 2 rows) or TGFBR3-B (cystoplasmic domain, green, bottom 2 rows). Reduced from ×20 (scale bar 100 μm). *B*, immunostaining for TGFBR3-A (green, 1:50), myosin (red) and nuclei (DAPI, blue) in E21 gubernaculum shows prominent soluble betaglycan collections in some fetuses. Reduced from ×10 (scale bar 200 μm).

regions of the gene, respectively. HapMap data (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/) demonstrate minimal LD between these 3 genomic regions. It is possible that the strength of the observed association for any 1 signal is limited by allelic heterogeneity and/or the unmeasured effects of variable environmental exposures.

Cryptorchidism is a complex disease, likely influenced by multilocus genetic, maternal and/or environmental factors. The genomic loci and pathways involved remain largely unknown, although many studies address this question in animal models. Gene targeting experiments in mice confirm requirement for *Rxfp2* and *Ar*, substantiate a role for Wnt and Notch signaling in gubernacular development, and confirm myogenesis as a key target of hormone action.[21,22]

TGFBR3/betaglycan is a biologically compelling risk locus for cryptorchidism because of its association with fetal Leydig cell development and myogenesis.[23,24] Betaglycan is a TGFβ coreceptor that facilitates or inhibits signaling, depending on the context.[25] The cytoplasmic domain facilitates

signaling by TGFβ2 or TGFβ1, while the soluble, cleaved ectodomain can modulate TGFβ signaling by sequestering these growth factors. Betaglycan is expressed in interstitial and peritubular myoid cells in the fetal testes of mice and humans.[26,27] *Tgfbr3* -/- mice exhibit disruption of tubular architecture and a transient delay in Leydig cell development, with reduced expression of *Insl3* and steroidogenesis genes.[24] It is noteworthy that inactivation of *Tgfβ2* in transgenic mice is associated with cryptorchidism without other external genital defects,[28] suggesting a primary gubernacular defect rather than global hormone deficiency. This and previous studies revealing expression of *Tgfbr3* and *Tgfb2* with increased expression in orl and wt males following androgen exposure support a role for TGFβ signaling in gubernaculum development.[18,20]

The present data suggest that membrane associated betaglycan is expressed throughout the fetal rat gubernaculum but most strongly in the outer mesothelial layer and in association with areas of developing muscle, and that focal increased accumulation of soluble betaglycan occurs in mesothelium and mesenchyme of orl gubernacula. Although we have not fully elucidated the genomic loci that confer susceptibility to cryptorchidism, our prior studies suggest that androgen signaling and muscle patterning are altered in the orl strain.[20,29]

Inconsistency in the pattern of association of *TGFBR3* with cryptorchidism may reflect various factors, notably insufficient sample size, allelic heterogeneity, population differences and/or phenotypic misclassification or variability. Regarding the latter, Dalgaard et al included subjects identified at birth or through medical records,[6] although it is unclear whether all had persistent cryptorchidism requiring surgery. In their discussion of the concept of "synthetic" association of GWAS signals with rare, causal variants in the same genomic region Dickson et al also hypothesized that even modest associations defined by GWAS may show loci that contain rare variants of larger effect.[30]

CONCLUSIONS

The present data warrant more intensive investigation to define potential causal variants and the role of betaglycan in reproductive development in experimental animal models. Hypotheses that should be addressed include the possibility that genomic loci participate in hormone signaling pathways that are also specific targets of environmental exposures, and that pleiotropic effects of genes may explain the co-occurrence of testicular germ cell defects (cancer and subfertility) and cryptorchidism.

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