Candidate Gene Association Study Implicates \( p63 \) in the Etiology of Nonsyndromic Bladder-Exstrophy-Epispadias Complex

Lihong Qi,1 Mei Wang,2 Garima Yagnik,3 Manuel Mattheisen,4 John P. Gearhart,5 Yegappan lakshmanan,6 Anne-Karolin Ebert,7 Wolfgang Rösch,7,8 Michael Ludwig,9 Markus Draaken10,11 Heiko Reutter11,12* and Simeon A. Boyadjiev5,13*

1Department of Public Health Sciences, School of Medicine, University of California, Davis, California
2Department of Population Health and Reproduction, University of California, Davis, California
3Section of Genetics, Department of Pediatrics, University of California Davis, Sacramento, California
4Department of Biomedicine, Aarhus University, Aarhus C, Denmark
5Department of Urology, The James Buchanan Brady Urological Institute, Johns Hopkins University, Baltimore, Maryland
6Children’s Hospital of Michigan, Department of Pediatric Urology, Detroit, Michigan
7Department of Pediatric Urology, St. Hedwig Hospital Barmherzige Brüder, Regensburg, Germany
8Department of Pediatric Urology, University Medical Center Regensburg, Germany
9Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany
10Department of Genomics, Life and Brain Center, University of Bonn, Bonn, Germany
11Institute of Human Genetics, University of Bonn, Bonn, Germany
12Department of Neonatology, Children’s Hospital, University of Bonn, Bonn, Germany
13Section of Genetics, Department of Pediatrics, University of California Davis, Sacramento, California

Received 23 March 2013; Revised 15 May 2013; Accepted 29 May 2013

BACKGROUND: Bladder-exstrophy-epispadias complex (BEEC) is a severe congenital anomaly that represents a spectrum of urological abnormalities where parts or all of the distal urinary tract fail to close during development. Multiple lines of evidence strongly suggested \( p63 \) as a plausible candidate gene. We conducted a candidate gene association study to further investigate the role of \( p63 \) in human BEEC.

METHODS: We conducted a family-based association study of \( p63 \) using 154 Caucasian patients with nonsyndromic BEEC and their unaffected parents. High throughput single nucleotide polymorphism (SNP) genotyping was carried out using Illumina’s Golden Gate Assay for 109 selected tagging SNPs localized within \( p63 \) with a minor allele frequency > 0.01. Individual and haplotype SNP transmission disequilibrium tests were conducted using Plink and Haploview, respectively. We also examined parent-of-origin effects using paternal asymmetry tests implemented in FAMHAP (http://famhap.meb.uni-bonn.de/index.html).

RESULTS: Nominal significant associations were identified between BEEC and six SNPs (rs17447782, rs1913720, rs6790167, rs9865857, rs1549369, rs4687100), and four haplotype blocks including or near these significant SNPs. Analysis of parent-of-origin effects showed significant results for seven SNPs (rs4118375, rs12696596, rs6779677, rs13091309, rs7642420, rs1913721, and rs1399774). None of these results remained significant after multiple testing correction.

CONCLUSION: The altered transmission of \( p63 \) variants in nonsyndromic BEEC patients may be suggestive of its involvement in the disease etiology. Further and large multi-institutional
collaborative studies are required to elucidate the role of p63 in nonsyndromic BEEC. Birth Defects Research (Part A) 00:000–000, 2013. © 2013 Wiley Periodicals, Inc.

**Key words**: association study; BEEC; bladder exstrophy; case–parent trio; candidate gene; epispadias; p63

**INTRODUCTION**

The bladder exstrophy-epispadias-complex (BEEC) is an anterior midline defect with variable expression ranging from isolated epispadias, to classical exstrophy of the bladder, to its most severe form, exstrophy of the cloaca also known as OEIS (omphalocoele, exstrophy of the bladder, imperforate anus, spinal defects) complex (Carey 2001; Boyadjiev et al., 2004; Ludwig et al., 2009). The incidence of the complete BEEC spectrum has been reported to be 1 in 10,000 live births with multiple surveys summarizing a male-to-female ratio of 2.4:1 (Ebert et al., 2009). Birth prevalence, including terminated pregnancies, for the specific subtypes have been estimated to be 1 in 117,000 in males and 1 in 484,000 in females for epispadias, 1 in 37,000 for classic bladder exstrophy (CBE) (Wiesel et al., 2005) and 1 in 200,000 to 1 in 400,000 for CE (Hurwitz et al., 1987). Although the BEEC can occur as part of a complex malformation syndrome, approximately 98.5% of cases are classified as nonsyndromic (Boyadjiev et al., 2004; Gambhir et al., 2008). Even though nonsyndromic BEEC is not life-threatening, it is associated with significant morbidity requiring multiple surgeries and compromises the quality of life of the affected.

Several reports of chromosomal aberrations associated with BEEC (Boyadjiev et al., 2005; Ludwig et al., 2009; Draaken et al., 2010; Lundin et al., 2010) and the reports of at least 30 families with multiple affected members (Reutter et al., 2003; Ludwig et al., 2009) may suggest that at least in some cases the malformation is determined by a single genetic mutation. Nevertheless, the general consensus in the field is that in the majority of patients the genetic basis of the BEEC appears to be multi-factorial (Boyadjiev et al., 2004; Ludwig et al., 2009). This is corroborated by the high but incomplete concordance rates of 62% among monozygotic twins as compared to the rate of 11% among dizygotic twins (Reutter et al., 2007) and increased recurrence risk among offspring of affected individuals of 1 in 70 (Shapiro et al., 1984). However, no causative genetic factor has been unequivocally identified in humans to date. Only two candidate genes, FGF10 (Fairbanks et al., 2004; Kruger et al., 2008) and p63 (Ince et al., 2002; Cheng et al., 2006; Ching et al., 2010) have been suggested by murine knockout models or analysis of human exstrophic bladders. While mice with a deletion of FGF10 partially resemble the phenotype of human CBE, mice with complete or partial deletion of p63 (p63+/− and ΔNp63−/−) resemble the full picture of human CE or CBE (Ince et al., 2002; Cheng et al., 2006). Based on these observations, a Sanger-sequencing based mutation screening of FGF10 and p63 was carried out in BEEC patients but could not identify any disease causing mutation (Kruger et al., 2008; Ching et al., 2010). However, in our study of the human p63 (Ching et al., 2010), we observed reproducible dysregulation of various p63 isoforms in bladder tissues of CBE patients, indicating altered gene expression of p63 in human BEEC. Furthermore, a genome-wide expression analysis independently implicated p63 as a contributing factor in the BEEC etiology (Qi et al., 2011). A recent study (Wilkins et al., 2012) independently corroborated our data (Ching et al., 2010) and also suggests that ΔNp63 is the dominant promoter in human tissue and its expression is significantly reduced in BEEC tissue in early bladder formation. The authors also showed that two insertion–deletion polymorphisms including a 12-base-pair deletion (rs6148242) and a homozygous 1bp insertion polymorphism (rs5855273) in the ΔNp63 promoter were associated with a highly increased risk for the development of nonsyndromic BEEC among Caucasians. These recent results and our own previous findings prompted us to conduct a case–parent trio based association study of the p63 gene using 154 patients with nonsyndromic BEEC patients and their unaffected parents, to further investigate the role of the p63 gene in the development of BEEC among Caucasians.

**MATERIALS AND METHODS**

**Study Sample**

We selected 154 case–parent trios with the child affected with nonsyndromic BEEC (proband). All probands were sporadic and the family histories were unremarkable for BEEC, neural tube defects, omphalocoele or any other BEEC associated congenital anomaly. Among the 154 probands, 16 had epispadias, 130 had classical exstrophy of the bladder and 8 had exstrophy of the cloaca. The overall male to female ratio in the complete sample was 99 to 55 (epispadias: 11 males and 5 females, classical exstrophy of the bladder: 86 males and 44 females, exstrophy of the cloaca: 2 males and 6 females).

A total of 120 case–parent trios were recruited from United States and 34 from Germany. All families were self-reported Caucasians. Written informed consent forms were approved by the Johns Hopkins Institutional Review Board (IRB) and the Ethics Committee of the Medical Faculty of the University of Bonn, and were obtained from all probands and their parents before study entry and sample collection. Blood was obtained from the probands and their parents through routine phlebotomy and in some cases saliva samples were collected with Scope oral mouthwash (Procter & Gamble Co.) or Oragene kit (DNA Genotek Inc., Kanata, Canada). Isolation of genomic DNA was carried out by a Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany) for the German samples and using the Puragen DNA purification kit (Gentra Systems) for the North American samples. Five micrograms of high molecular weight genomic DNA from each individual was used for genotyping.

**Genotyping**

We selected 109 tagging single nucleotide polymorphisms (SNPs) localized within p63 with a minor allele...
The distribution of SNPs along the 26 kb of genomic DNA spanned by the p63 gene was random based on the availability of tagging SNPs with the largest distance between two neighboring SNPs being 13 kb. Genomic DNA samples were diluted and quantified on a VeraQuant DNA Processor (Applied Biosystems). Genomic DNA samples were processed in multiples of 96 (5 μl/sample at a concentration between 50 and 100 ng/μl) and analyzed for all SNPs. The assay generates hundreds of templates with specific target and address sequences using allele-specific extension, followed by ligation and amplification with universal primers. Fluorescent products were hybridized to precoded beads and excited by lasers in Illumina’s BeadXpress Reader. Signal intensities were quantified and matched to specific alleles using GenomeStudio software v2011.1. Manual reclustering of homozygous and heterozygous readers using TDT Analysis and Analysis of Parent-of-Origin Effects of Individual SNPs.

<table>
<thead>
<tr>
<th>SNP</th>
<th>OR</th>
<th>p-value</th>
<th>Permuted p-value</th>
<th>POO* p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4118375</td>
<td>0.81</td>
<td>0.39</td>
<td>1.00</td>
<td>0.03</td>
</tr>
<tr>
<td>rs12696596</td>
<td>1.05</td>
<td>0.75</td>
<td>1.00</td>
<td>0.05</td>
</tr>
<tr>
<td>rs17447782</td>
<td>0.55</td>
<td>0.04</td>
<td>0.93</td>
<td>0.48</td>
</tr>
<tr>
<td>rs1913720</td>
<td>0.71</td>
<td>0.04</td>
<td>0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>rs5642420</td>
<td>0.72</td>
<td>0.22</td>
<td>1.00</td>
<td>0.05</td>
</tr>
<tr>
<td>rs1913721</td>
<td>1.10</td>
<td>0.56</td>
<td>1.00</td>
<td>0.04</td>
</tr>
<tr>
<td>rs1399774</td>
<td>0.75</td>
<td>0.16</td>
<td>1.00</td>
<td>0.001</td>
</tr>
<tr>
<td>rs6790167</td>
<td>1.40</td>
<td>0.04</td>
<td>0.92</td>
<td>0.31</td>
</tr>
<tr>
<td>rs9865857</td>
<td>0.68</td>
<td>0.02</td>
<td>0.78</td>
<td>1.00</td>
</tr>
<tr>
<td>rs1543969</td>
<td>0.69</td>
<td>0.04</td>
<td>0.93</td>
<td>0.38</td>
</tr>
<tr>
<td>rs4687100</td>
<td>0.65</td>
<td>0.02</td>
<td>0.78</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Family-based association testing was conducted using the transmission disequilibrium test (TDT) in Plink. Odds ratio (OR) and p-values were presented. The nominally significant SNP association is bolded. Corrected p-values following 10,000 permutations are also indicated.

The p-values for parent-of-origin (POO) effects examined using the paternal asymmetry test implemented in FAMHAP. SNP, single nucleotide polymorphism; OR, odds ratio.

Statistical Analysis
We analyzed genotype data from 154 case–parent trios. Pair-wise linkage disequilibrium heatmap was created using Haploview (http://www.broadinstitute.org/haplov) (Barrett et al., 2005). The Hard-Weinberg equilibrium test was performed in unrelated subjects (i.e., parents in each trio), and SNPs with p-value < 0.001 were excluded from analyses. We conducted individual SNP transmission disequilibrium tests (TDT) (Spielman et al., 1993) using Plink v1.07 with 10,000 permutations (the option mperm = 10,000) (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007), excluding SNPs with Mendelian error rates ≥ 0.05, and families with Mendelian error rate ≥ 0.1. Multiple comparisons were corrected by generating empirical p values based on gene-dropping permutation using the max (T) procedure implemented in Plink. Haplotype blocks were constructed and haplotype-based TDT was conducted using Haploview, and the multiple testing was corrected with 1000-permutations in Haploview. All TDT tests were two-sided and a p value of < 0.05 was considered as statistical significant after correcting for multiple comparisons.

We also examined parent-of-origin effects using the paternal asymmetry test (Weinberg, 1999) implemented in FAMHAP (http://famhap.med.uni-bonn.de/index.html). Multiple testing was corrected using the Bonferroni method.

RESULTS
A total of 109 SNPs were genotyped and the overall genotyping rate in individuals was 99.6%. Two SNPs (rs7631262 and rs7653848) were excluded from analyses based on the Hard-Weinberg equilibrium test (p-value < 0.001). Supplementary Table 1, which is available online, presents the MAF of the 107 SNPs included in analyses and the p values of their Hard-Weinberg equilibrium tests. The lowest MAF was 0.01 (rs6763902) and the highest was 0.49 (rs9835923, rs9881595 and rs12107036).

In the individual SNP TDT analysis, six SNPs (rs17447782, rs1913720, rs17447782, rs7690167, rs9865857, rs1543969, rs4687100) showed nominally statistically significant results (p-value < 0.05) (Table 1). After correction for multiple comparisons, none of the SNPs was significant. Analysis of parent-of-origin effects showed significant results for seven SNPs (rs17447782, rs12696596, rs9865857, rs1543969, rs4687100) (p-value < 0.05; Table 1), but none remained significant after correction for multiple testing (results not shown).

RESULTS
A total of 109 SNPs were genotyped and the overall genotyping rate in individuals was 99.6%. Two SNPs (rs7631262 and rs7653848) were excluded from analyses based on the Hard-Weinberg equilibrium test (p-value < 0.001). Supplementary Table 1, which is available online, presents the MAF of the 107 SNPs included in analyses and the p values of their Hard-Weinberg equilibrium tests. The lowest MAF was 0.01 (rs6763902) and the highest was 0.49 (rs9835923, rs9881595 and rs12107036).

In the individual SNP TDT analysis, six SNPs (rs17447782, rs1913720, rs7690167, rs9865857, rs1543969, rs4687100) showed nominally statistically significant results (p-value < 0.05) (Table 1). After correction for multiple comparisons, none of the SNPs was significant. Analysis of parent-of-origin effects showed significant results for seven SNPs (rs17447782, rs12696596, rs9865857, rs1543969, rs4687100) (p-value < 0.05; Table 1), but none remained significant after correction for multiple testing (results not shown).

RESULTS
A total of 109 SNPs were genotyped and the overall genotyping rate in individuals was 99.6%. Two SNPs (rs7631262 and rs7653848) were excluded from analyses based on the Hard-Weinberg equilibrium test (p-value < 0.001). Supplementary Table 1, which is available online, presents the MAF of the 107 SNPs included in analyses and the p values of their Hard-Weinberg equilibrium tests. The lowest MAF was 0.01 (rs6763902) and the highest was 0.49 (rs9835923, rs9881595 and rs12107036).

In the individual SNP TDT analysis, six SNPs (rs17447782, rs1913720, rs7690167, rs9865857, rs1543969, rs4687100) showed nominally statistically significant results (p-value < 0.05) (Table 1). After correction for multiple comparisons, none of the SNPs was significant. Analysis of parent-of-origin effects showed significant results for seven SNPs (rs17447782, rs12696596, rs9865857, rs1543969, rs4687100) (p-value < 0.05; Table 1), but none remained significant after correction for multiple testing (results not shown).

DISCUSSION
We performed a comprehensive candidate gene association study using tagging SNPs covering the whole p63 locus to evaluate the role of p63 in the development of human nonsyndromic BEEC among Caucasians. We used a case–parent trio design to avoid undetected ethnic stratification as a cause of false positive results. In our
sample of 154 trios, we observed significant transmission distortion for six (rs17447782, rs1913720, rs6790167, rs9665857, rs1543969, rs4687100) of 107 individual markers before correction for multiple testing (Table 1). However, the p-values fell short of nominal significance after correction for multiple testing. Analysis at the level of haplotypes resulted in four haplotype blocks showing significant transmission distortion but they became not significant after correction of multiple testing.

Recently, Wilkins et al., (2012) reported two polymorphisms in the ΔNp63 promoter (rs6148242, rs5855273) to be strongly associated with an increased risk of BEEC among Caucasian patients. Although we found several SNPs in the p63 gene that showed altered transmission from the parents to their BEEC offspring, the results lost statistical significance after correction for multiple testing. Considering these results and our own previous observations of dysregulation of various p63 isoforms in bladder tissues of patients with classical exstrophy of the bladder (Ching et al., 2010; Qi et al., 2011), our present sample size might have not been large enough to reach statistical significance after multiple testing correction. To

Table 2
Haplotype TDT Analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Haplotype</th>
<th>p-value</th>
<th>Permutated p-value</th>
<th>SNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>H15</td>
<td>11</td>
<td>0.06</td>
<td>0.95</td>
<td>rs13059768</td>
</tr>
<tr>
<td>H15</td>
<td>22</td>
<td>0.46</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H15</td>
<td>12</td>
<td>0.02</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>H16</td>
<td>21212</td>
<td>0.10</td>
<td>1.00</td>
<td>rs1913720</td>
</tr>
<tr>
<td>H16</td>
<td>11121</td>
<td>0.57</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H16</td>
<td>11111</td>
<td>0.87</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H16</td>
<td>22211</td>
<td>0.20</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H16</td>
<td>21211</td>
<td>0.88</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H16</td>
<td>11211</td>
<td>0.03</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>H17</td>
<td>12</td>
<td>0.02</td>
<td>0.74</td>
<td>rs6790167</td>
</tr>
<tr>
<td>H17</td>
<td>21</td>
<td>0.04</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>H17</td>
<td>11</td>
<td>0.90</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H19</td>
<td>12</td>
<td>0.04</td>
<td>0.78</td>
<td>rs1554131</td>
</tr>
<tr>
<td>H19</td>
<td>21</td>
<td>0.92</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>H19</td>
<td>11</td>
<td>0.06</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

Haplotype blocks were constructed based on the default algorithm in Haploview. Nominally significant haplotype associations with BEEC and significant SNPs in the individual SNP TDT are bolded. Corrected p-values following 1000 permutations are also indicated.

BEEC, bladder extrophy-epispadias-complex; SNPs, single nucleotide polymorphisms; TDT, transmission disequilibrium tests.
address whether the lack of statistical significance after correction of multiple testing may be attributed to limited sample size, we calculated the power for the individual SNP TDT analysis using the Genetic Power Calculator (Purcell et al., 2003) assuming an odds ratio of no more than 1.8. Our calculation yielded a power of 0.87 at $\alpha = 0.05$ level, suggesting that our sample size provides enough power to detect the effect at the significant level of 0.05. But the power reduced to 0.36 when using a more stringent significance level of 0.0005 ($=0.05/107$) to account for multiple testing, supporting the hypothesis that the failure to reach statistical significance after multiple testing correction for these SNPs might have been due to the moderate sample size of our cohort.

In summary, we observed significant transmission distortion for six SNPs before correction for multiple testing in our candidate gene study of the \textit{p63} locus among Caucasian patients. The altered transmission of \textit{p63} variants in nonsyndromic BEEC patients may be suggestive of its involvement in the disease etiology. Further and large multi-institutional collaborative studies are required to elucidate the role of \textit{p63} in nonsyndromic BEEC.

**ACKNOWLEDGMENTS**

We thank all of the patients and their families for participating in this study. M.L., A.K.E., W.R., M.D., and H.R. are members of the “Network for the Systematic Investigation of the Molecular Causes, Clinical Implications, and Psychosocial Outcome of Congenital Uro-Rectal Malformations (CURE-Net”)”. The authors have no conflicts of interest to declare. S.A.B. is partially funded through a Children’s Miracle Network Endowed Chair, Children’s interest to declare. S.A.B. is partially funded through a

**REFERENCES**


