A Locus for Renal Malformations Including Vesico-Ureteric Reflux on Chromosome 13q33–34

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Congenital anomalies of kidney and urinary tract (CAKUT), including vesico-ureteric reflux (VUR), are major causes of ESRD in childhood. Herein is reported evidence for a locus on 13q33q34 associated with CAKUT. Deletion mapping of chromosome 13q was performed in four children with CAKUT using 31 microsatellite markers on peripheral blood genomic DNA that was obtained from the patients and their parents. mRNA expression of the positional candidate genes was compared with sequences in electronic databases in silico and also studied in adult and fetal mouse kidneys using reverse transcription–PCR. The children (three girls; age range 5 to 17 yr) had varying severity of developmental delay and other organ system involvement. The spectrum of CAKUT included high-grade VUR (n = 2), renal dysplasia (n = 2), and hydronephrosis (n = 1). Both the children with VUR had evidence of renal failure with one of them developing ESRD. Deletion mapping identified a 7-Mb critical region flanked by markers D13S1311 and D13S285. There are 33 genes (12 known; 21 computer predicted) in this region. In silico expression studies showed matches for 14 of these genes in the kidneys and 10 in the bladder expressed sequenced tags databases. Mouse kidney studies showed that of the 24 genes examined, several had variable expression through the different stages of renal development, whereas five of the genes were not expressed at all. Herein is reported a new locus on chromosome 13q33q34 that can be associated with VUR with several genes showing mRNA expression patterns that suggest their potential for involvement in renal/urinary tract developmental anomalies.


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The software programs described in this study are available free of charge by contacting the authors. We are currently enrolling additional patients with 13q deletion for our studies and would appreciate contact from interested health care providers and patient families. The contact information is provided below.

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recently reported a CAKUT-associated locus on 13q12–22 (22). We now describe the results of cyto- and molecular genetic studies to identify a second region on chromosome 13 that is located on 13q33–34 and is associated with CAKUT including severe VUR and RN.

Materials and Methods
The study was approved by the Human Rights Committee and the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh. Informed consent was obtained from the parents or legal guardians of the children described in the study. The patients were investigated by various biochemical and radiologic tests, including ultrasonography, vesico-cysto-urethrography (VCUG), intravenous pyelography, computerized tomography and magnetic resonance imaging.

Cytogenetic and Deletion Mapping Studies
GTG banding was performed on peripheral blood lymphocytes from the proband and the parents using standard protocols as described previously (22,23). Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol-chloroform extraction for deletion mapping as described previously (22,24).Thirty-one microsatellite markers that spanned the 13q region were examined. Primer pairs were obtained commercially (Research Genetics, Huntsville, AL) or synthesized using primer sequences that are available in UniSTS database from Integrated DNA Technologies (Corvalle, IA). Microsatellite markers were analyzed after amplification by PCR. The PCR reactions were performed as described previously (22,24,25). PCR products were diluted 1:1 with loading buffer (95% deionized formamide, 20 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue), heat-denatured, and electrophoresed through 7% polyacrylamide gel that contained 5.6 M urea and 32% formamide for 3 h at 55°C. Gels were exposed to x-ray films at −80°C. Genotypes were scored visually, and deletion was mapped by comparing the genotypes of parents with those of the probands. The markers used were D13S787, D13S1493, D13S894, D13S626, D13S784, D13S791, D13S889, D13S891, D13S318, D13S776, D13S580, D13S265, D13S281, D13S167, D13S793, D13S154, D13S1252, D13S159, D13S779, D13S1267, D13S317, D13S1574, D13S280, D13S1311, D13S796, D13S286, D13S895, D13S1315, D13S148, D13S261, and D13S285.

Gene Expression Studies
Gene Expression Analyses In Silico. We used computational techniques that were developed in our laboratory to perform in silico analyses to study the expression of 13q candidate genes in kidneys and urinary tract. We first created various local BLAST searchable databases for all of the expressed sequenced tags (EST) and reference sequences (RefSeq) that are expressed in either kidneys or urinary tract (renal and urinary tract transcriptome). The sequences were retrieved from the National Center for Biotechnology (NCBI) BLAST ftp site (http://ftp.ncbi.nlm.nih.gov/blast/db/) using a set of queries and programs. A program called nucSearch.pl was written in PERL script to submit queries to NCBI that retrieved the GenInfo Identifier (GI) numbers for various EST. Unique queries were used to create different BLAST searchable databases. For example, to create the database for genes that are expressed in lower urinary tract (ureter, bladder, and trigone transcriptome), we used queries “ureter AND mRNA,” “trigone AND mRNA,” and “urinary bladder AND mRNA.” To create the “kidney transcriptome” database, the queries were “sapiens AND kidney AND mRNA,” “norvegicus AND kidney AND mRNA,” and “musculus AND kidney AND mRNA.” Each set of files then was concatenated and used to create their respective databases using the NCBI “formatdb” program with the -f and -l options to alias the entire EST and RefSeq database and restrict the search to only those GI numbers that were a result of the queries. A second program, called g japonica, also written in PERL, was used to submit the GI numbers sequentially to NCBI (http://www.ncbi.nlm.nih.gov/entrez/?db = Nucleotide) and to retrieve the FASTA sequence corresponding to the numbers. The retrieved FASTA sequences were formatted into four local BLAST databases that were created for the renally expressed genes of humans, mice, and rats and also a common database for all of the genes that are expressed in ureter, bladder, and trigone. All of the GI numbers of EST that lie on chromosome 13 between the two given microsatellite markers were retrieved from the NCBI. These FASTA sequences then were compared against various local BLAST databases separately using the -i option. The output files then were run through a parser program (called “BLAST Result Parser” available at http://www.ncbi.nlm.nih.gov/). Gene Expression in Mouse Kidneys. Primers for amplifying cDNA were synthesized (Integrated DNA Technologies, Coralville, IA) for the genes that lie in the 13q33q34 candidate region, and their expression was studied in FVB mice fetal (embryonic day of life 16 [E16]), neonatal, and adult (21 d old) kidneys by reverse transcription–PCR (26). FVB mice (The Jackson Laboratory, Bar Harbor, ME) are so named because they carry the Fv1b allele for sensitivity to the B strain of Friend leukemia virus. This strain is useful for many fetal/neonatal experiments as they have a vigorous reproductive performance with large litters. All reagents, including enzymes, were obtained from Invitrogen Corp. (Carlsbad, CA). Briefly, 2 μg of total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase with oligo dT priming. Amplification of cDNA was performed (35 to 40 cycles) by PCR using the following condition: The cycles were initiated by denaturing the DNA at 94°C for 30 s, followed by annealing reaction for 30 s at 58°C and extending at 72°C for 45 s. The PCR products were resolved on 2% agarose gel and photographed using KODAK EDAS-2 gel documentation system (Eastman Kodak, New Haven, CT).

Results
We identified five children with 13q deletion in the 13q33–34 region. Four of these five children had a renal anomaly identified by renal ultrasound or clinical history, and incomplete clinical data were available on one child. Further studies were done on the remaining three patients and are presented here. One of the patients (patient 1) attended the clinics at Children’s Hospital of Pittsburgh, and the others were identified through the Chromosomal Deletion Outreach organization (www.chromodisorder.org). Table 1 shows a summary of findings in the children studied, including the karyotype findings identified with GTG banding. Patient 1 had the most severe renal involvement with CAKUT as well as ESRD that led to dialysis. All of the children had involvement of other organ systems besides the kidneys/urinary tract and had varying severity of developmental delay.

Patient 1
This 17-yr-old girl in foster care presented with seizure disorder and mild developmental delay at the age of 12 yr. Investigations showed total calcium 4.3 mg/dl with ionized calcium 0.68 mmol/L, phosphate 7.7 mg/dl, HCO3 18 mEq/L, and serum creatinine 10.5 mg/dl, suggesting end-stage renal failure. Correction of hypocalcemia ameliorated the seizure disorder. A VCUG showed outpouching of bladder dome, presumed
to be urachal remnant, a single left-sided bladder diverticulum, and a right sided grade 4 VUR (Figure 1, A and B). A renal ultrasound showed left echogenic kidney that measured 5 cm in length (Figure 1C). The right kidney was caudally displaced and hyperechoic and measured 5.5 cm in length. Urinalysis showed 1+ proteinuria with two to three white cells per high-power field and no red blood cells. A computerized tomography scan that was obtained at 2 yr of age had shown no structural malformations of brain. She was found to have a 13q32.3 deletion (Figure 2A). She began dialysis soon after initial presentation and has been seizure-free since initiation of dialysis except for one brief episode, lasting 30 s, approximately 18 mo after initiating hemodialysis.

**Patient 2**
This 5-yr-old girl was born full term. Her postnatal course was complicated by hypotonia, feeding difficulties, seizure disorder, and poor weight gain. She had mild facial dysmorphism and developmental delay (Figure 1D). She was found to have chromosome 13q33q34 deletion at 8 mo of age (Figure 2B). Subsequently, she developed recurrent UTI, including several episodes of severe pyelonephritis. A renal ultrasound at 2 yr of age showed slightly small kidneys with normal shape and echogenicity. A VCUG showed bilateral VUR (grade 4 to 5). A magnetic resonance imaging scan showed no structural malformations of brain. She underwent a right cutaneous ureterostomy and left uretero-ureterostomy at 30 mo of age. Her serum creatinine at 3 yr of age was elevated at 1.1 mg/dl.

**Patient 3**
This 14-yr-old girl was born full term and received a diagnosis of chromosome 13q32q34 deletion at 5 wk of age. She had recurrent UTI until 3 yr of age and required frequent antibiotic courses. A renal ultrasound that was performed at 15 mo of age showed grade 2 hydronephrosis of the left kidney with dilatation of the left proximal ureter. The left kidney measured 5.1 cm, and the right measured 5.3 cm in length at that time. She was not found to have VUR on a VCUG that was performed at approximately the same time, but it did show a large-capacity bladder with a volume of 250 ml and significant residual volume. She also had dysfunctional urinary voiding until approximately 3 yr of age. Serum blood urea nitrogen and creatinine that were obtained at 10 yr of age were 11 and 0.7 mg/dl, respectively. Subsequent renal ultrasound at 12 yr of age (weight 40 kg) showed resolution of urinary tract dilation but showed small-sized kidneys (left 7.5 cm and right 7.8 cm) with normal echogenicity. She also had hematuria and proteinuria on urinalyses (generally performed with UTI episodes), which resolved. She developed enuresis at 13 yr of age, which spontaneously resolved during the next year. Her other problems include a T7 hemivertebra and malformed vertebrae from T8 to T12 and a clinically visible spinal curvature and scoliosis at T12/L1 level. She also has an abnormal anteriorly placed anal opening and severe constipation. She underwent an Achilles tendon release at the age of 3 yr and can walk and ambulate well now. She has had significant cognitive delay and currently functions at a 2.5- to 3-yr-old level (Figure 1E). She has no spoken language except for a few words but has good motor skills.

**Cytogenetic and Deletion Mapping Studies**
The details of the karyotype for patients 1 and 2 as well as an ideogram of the deleted region is shown in Figure 2, C through E. Table 2 shows the details of deletion mapping studies. The PCR product sizes for each marker pair and the visually ascertained alleles for each individual studied (arbitrarily labeled alphabetically) are shown. The deleted chromosomal region for each patient is shown in boldface type in Table 2. The order of the markers is based on the May 2004 assembly of the human genome available through University of California Santa Cruz genome browser (http://genome.ucsc.edu). The genetic distances for the markers were derived from the genetic location database (ftp://cedar.genetics.soton.ac.uk/pub/chrom13/map.html). Patient 1 had only maternal blood sample available, and deletion studies were performed using a smaller
number of microsatellite markers because of limited DNA availability as she was in foster care. Paternal blood sample was not available for patient 1, and her deletion mapping studies were interpreted to show a deletion that extended from D13S779 to D13S285 because this region coincided with the deleted region that was visible on the karyotype. She had apparent loss of paternal alleles. Patient 2 showed a deletion from D13S1311 to at least D13S285 with a loss of paternal alleles. The distal limit of the deletion was not mapped for patient 2. Patient 3 also showed a deletion that extended from D13S779 to D13S285 with loss of maternal alleles. Our studies suggest existence of a critical region in the 13q33q34

Figure 1. Clinical findings of patients with 13q deletion. (A) Vesico-cysto-urethrography (VCUG) of patient 1 showing a right-sided grade 4 vesico-ureteric reflux (VUR). (B) An outpouching of bladder dome, presumed to be urachal remnant is shown; a single left-sided bladder diverticulum is shown in inset. (C) Renal ultrasound showing echogenic kidneys. The left kidney measured 5 cm in length, and the right kidney measured 5.5 cm in length. (D and E) Mild facial dysmorphic features of patient 2 (D) and patient 3 (E). The facial pictures are published with signed consent of the parents of both children.
area that is involved in the development of CAKUT, especially VUR. The region is defined by markers D13S1311 proximally and D13S285 distally extending approximately 7 Mb.

**Gene Expression Studies**

We identified 33 genes (12 known; 21 computer predicted) in the critical region based on the May 2004 assembly of human genome. *In silico* gene expression comparison with rodent and human renal and urinary bladder EST databases was performed to identify whether any of the 13q candidate genes have been identified to have renal expression. We downloaded a total of 472,765 unique sequences to create the three renal "transcriptomes" for human, mouse, and rat using query terms described earlier. The database for genes that are expressed in urinary bladder, ureter, and trigone had 123,802 sequences. We performed local BLAST match for the 33 genes in the 13q33 region against these databases. All of the genes with a significant match (an e value score of $<10^{-10}$) and the match scores are presented in Figure 3; in this figure, a higher score represents a better match. There were 11 matches in the human kidney transcriptome database, nine of which were near perfect. There were 12 matches in the mouse kidney, five in the rat kidney, and 10 in the bladder databases. The matches in the nonhuman and bladder databases had lower similarity scores than the human database matches, and also

![Figure 2.](image_url)
four matches were nonoverlapping with the human renal transcriptome. These *in silico* expression studies, therefore, showed matches for a total of 14 genes in the kidneys and 10 in the bladder EST databases.

Finally, we studied the expression patterns of all of the known genes and a majority of the computer-predicted genes in mouse renal tissue. A total of 24 13q33 candidate genes and two housekeeping genes (*H9252*-actin and glyceraldehyde-3-phosphate dehydrogenase) were analyzed (26 total). We designed primers to amplify the cDNA for all of these genes (shown in Figure 3). The primers were designed to amplify regions of genes/EST that were homologous for both human and mouse sequences (if available). The reverse transcription–PCR expression profiles in mouse kidneys of these genes are summarized in the last columns of Figure 3 and shown in Figure 4. Several of the genes had bands at slightly different sizes than expected (marked by an asterisk in Figure 3). This could be explained by alternate splicing or incomplete characterization of the computer-predicted genes in the GenBank. Overall, these mouse kidney expression studies generally confirmed the expression pattern identified in the *in silico* gene expression analyses. Nine genes had renal expression that was not identified by the *in silico* analyses. In addition, it showed that of the 24 genes examined, several had variable expression through the different stages of renal development. Five of the genes were not expressed at all in kidneys at any stage. Although one possible explanation for this observation is that the PCR primers did not work at all for those genes, but given that the housekeeping genes (*H9252*-actin and glyceraldehyde-3-phosphate dehydrogenase) were well expressed at the same time in the same tissues, it is likely that these genes truly are not expressed in developing or mature mouse kidney. Also, two of the genes had expression in embryonic or neonatal kidney but not in the adult kidney.

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<th>Position (Kb)</th>
<th>PCR Product Size</th>
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<th>Patient 2 (VUR)</th>
<th>Patient 3 (Hydronephrosis)</th>
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Boldface type denotes deleted alleles of chromosome 13q microsatellite markers.
Figure 3. Candidate genes in the 13q33q34 region and their expression in kidneys and urinary tract. *Size discrepancy between predicted and observed size of PCR fragment (possible alternate splicing).

Figure 4. Candidate gene expression from 13q33 location in fetal, neonatal, and adult mouse kidneys. The numbers on top of each lane refer to the genes in the last column of Figure 3, and the last lane shows a DNA ladder.
Discussion
Congenital renal anomalies, including VUR, are a major cause for morbidity in the pediatric age group and are becoming a significant cause of ESRD in adulthood (1,4–6). Previous studies have shown that VUR can occur in families, and genetic factors likely play an important role in the pathogenesis (9–12,16). Different modes of inheritance of VUR have been suggested, including a dominant single gene and polygenic inheritance (27–29). Although cases of 13q deletion syndrome have been identified occasionally with renal malformations, VUR and ESRD previously have not been associated with 13q deletion (30–33). Also, before our studies, no information was available on the critical regions of 13q associated with renal development. We had previously reported association of interstitial deletion of 13q12q22 with CAKUT (22) and now present evidence for a second locus on 13q33q34 associated especially with severe VUR, RN, and ESRD.

Our studies suggest presence of at least one gene in the 7-Mb region on 13q33–34 that is likely to be responsible for in utero renal and urinary tract development. We hypothesized that for a gene to be involved in CAKUT, it has to be expressed in the kidney. Therefore, we conducted in silico and mouse kidney mRNA expression studies of all of the genes in this region. This chromosomal region has 33 genes (including some computer predicted) and include several interesting genes, such as collagen IV subunits 1 and 2 (COL4A1 [MIM 120130] and COL4A2 [MIM 120090]). COL4A1 and A2 are not currently associated with any human renal disease but showed strong renal expression. As other COL4 chains are associated with Alport syndrome and its variants (34,35), it is intriguing to speculate a role for these genes in renal development. The mRNA expression studies also identified several genes to have no renal or urinary tract expression, therefore making them less likely to be involved in renal ontogeny. In contrast, the genes with a high renal expression, especially in the embryonic phase of development, would be good candidates for further exploring their role in CAKUT.

There is considerable variability in the renal and extrarenal manifestations of 13q deletion syndrome (32,33). It generally is thought that 13q deletions have three major phenotypic associations. Proximal 13q deletions (especially those involving 13q14) show mild to moderate developmental delay and an almost 80% risk for development of retinoblastoma (31). More distal deletions, involving 13q32, have associated holoprosencephaly, severe developmental delay, growth deficiency, and limb anomalies. Finally, the most distal deletion, involving 13q33q34, is associated with severe developmental delay but no gross malformation or growth deficiency (30,31). We identified children with severe VUR and renal failure from this last group that involves 13q33q34. Our previous study had identified a region on 13q12q22 that is centromeric to the current critical region on 13q33q34, which was associated with CAKUT manifesting as obstructive uropathy but not VUR (22). There is no apparent overlap between these two distinct 13q loci associated with CAKUT. Taken together with our earlier report of a CAKUT locus on 13q12–22 region, we believe that at least two loci for genes on chromosome 13q are associated with renal/ genitourinary development in utero.

CAKUT most likely is a polygenetic condition. In the only genome-wide study of seven families with nonsyndromic VUR, a linkage to chromosome 1 was reported by Feather et al. (20). It is interesting that two of the seven pedigrees in that report did not link to this locus, suggesting genetic heterogeneity for VUR. Recently, Sanna-Cherchi et al. (21) analyzed another seven large European families with VUR. They studied markers on chromosomes 1p13, 3p12, 6p21, 10q26, and 19q13, but no linkage to any of these loci was found. These data demonstrate substantial genetic heterogeneity of VUR, and our findings of a 13q33 locus lend further credence to their observation. There may be additional but only moderate support for a 13q33 locus for renal diseases if we analyze some of the published reports more carefully (20,36). It is interesting that Feather et al. (20) in their report also identified 12 additional possible loci for VUR with a P < 0.05 including a locus on 13q. The cumulative parametric logarithm of odds (LOD) score for the 13q locus (in the region of 96 to 113 cm) in that study was 2.08 (nonparametric linkage score = 2.37; P = 0.02) when all of the families that were not linked to chromosome 1 were analyzed. Although these LOD scores were small, their data are at least suggestive of a possible locus on chromosome 13 that seems to overlap with the locus described in this report. Finally, in an attempt to map the genes that predispose to the common causes of ESRD, a genome-wide scan in 1023 individuals with chronic kidney disease from 483 black families was performed by Freedman et al. (36). Their results showed a modest evidence of linkage to 13q33.3 near D13S796 on multipoint nonparametric linkage analysis (LOD = 1.72). Incidentally, marker D13S796 is in the middle of the critical 13q33 region found in our study (Table 2). Although there are no data available on the presence of VUR in the Freedman et al. study, it is conceivable that gene(s) in this region may contribute to both renal function and anatomy. VUR was documented recently to be not an infrequent finding in adults with incidentally diagnosed hypertension from an Indian cohort of patients (6). This locus therefore may be worthy of further examination in both syndromic and nonsyndromic VUR.

Conclusion
We report association of interstitial deletion of 13q33q34 with VUR and ESRD. This region contains several genes that show renal expression, and these would be good candidate genes for further study. More patients or pedigrees with 13q deletion and with inherited CAKUT/VUR will need to be tested to identify the role of this region in both syndromic and nonsyndromic VUR.

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References