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GENETIC DISORDERS – DEVELOPMENT

Lack of major involvement of human uroplakin genes in vesicoureteral reflux: Implications for disease heterogeneity

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Lack of major involvement of human uroplakin genes in vesicoureteral reflux: Implications for disease heterogeneity.

Background. Primary vesicoureteral reflux (VUR) is a hereditary disorder characterized by the retrograde flow of urine into the ureters and kidneys. It affects about 1% of the young children and is thus one of the most common hereditary diseases. Its associated nephropathy is an important cause of end-stage renal failure in children and adults. Recent studies indicate that genetic ablation of mouse *uroplakin (UP) III* gene, which encodes a 47 kD urothelial-specific integral membrane protein forming urothelial plaques, causes VUR and hydronephrosis.

Methods. To begin to determine whether mutations in *UP* genes might play a role in human VUR, we genotyped all four *UP* genes in 76 patients with radiologically proven primary VUR by polymerase chain reaction (PCR) amplification and sequencing of all their exons plus 50 to 150 bp of flanking intronic sequences.

Results. Eighteen single nucleotide polymorphisms (SNPs) were identified, seven of which were missense, with no truncation or frame shift mutations. Since healthy relatives of the VUR probands are not reliable negative controls for VUR, we used a population of 90 race-matched, healthy individuals, unrelated to the VUR patients, as controls to perform an association study. Most of the SNPs were not found to be significantly associated with VUR. However, SNP1 of *UP Ia* gene affecting a C to T conversion and an Ala7Val change, and SNP7 of *UP III* affecting a C to G conversion and a Pro154Ala change, were marginally associated with VUR (both $P = 0.08$). Studies of additional cases yielded a second set of data that, in combination with the first set, confirmed a weak association of UP III SNP7 in VUR ($P = 0.036$ adjusted for both subsets of cases vs. controls).

Conclusion. Such a weak association and the lack of families with simple dominant Mendelian inheritance suggest that missense changes of uroplakin genes cannot play a dominant role in causing VUR in humans, although they may be weak risk factors contributing to a complex polygenic disease. The fact that no truncation or frame shift mutations have been found in any of the VUR patients, coupled with our recent finding that some breeding pairs of UP III knockout mice yield litters that show not only VUR, but also severe hydronephrosis and neonatal death, raises the possibility that major uroplakin mutations could be embryonically or postnatally lethal in humans.

Key words: vesicoureteral reflux (VUR), hydronephrosis, uroplakin, urothelium.

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Primary, nonsyndromic, vesicoureteral reflux (VUR; OMIM, 193000) is characterized by the retrograde flow of urine from the urinary bladder to the ureters and kidneys. This congenital anomaly affects about 1% of young children and is thus one of the most common congenital anomalies affecting humans [1]. VUR is thought to be associated with the abnormally lateral insertion of the ureters into the bladder leading to an enlarged ureteral orifice and a shortening of the segment of the ureter, which normally traverses obliquely through the bladder wall. Individuals with VUR are prone to urinary tract infection (UTI); indeed, up to 30% of children presenting with such infections have VUR [1]. The reflux of infected urine into the kidney can cause acute pyelonephritis and subsequent scarring; such "reflux nephropathy" is an

important cause of end-stage renal failure in both children and adults, accounting for 10% to 15% of all patients who require long-term dialysis and/or renal transplantation [2, 3].

VUR has a strong hereditary component, with a 30- to 50-fold increase in incidence in first-degree relatives of VUR probands [4–7]. Indeed, systematic screening with micturating cystography of young, first-degree relatives of VUR index cases has revealed that up to 50% of siblings and offspring are affected by VUR [5, 6]. Other investigators have identified several kindreds with dominant inheritance of VUR and associated neprhopathy [8, 9]. As some of the kidney damage associated with VUR is secondary and hence potentially preventable by prophylactic antibiotic treatment and/or surgical intervention, there is a clear need for the early detection of this condition. In addition, currently VUR is definitely diagnosed in young children by voiding cystourethrography or direct radionuclide micturating cystography, which are invasive and costly; the identification of genes involved in VUR could lead to the development of genetic screening for infants. Feather et al [9] has demonstrated that some families link to a locus on 1p13; however, the same study demonstrated that VUR was genetically heterogeneous, with two families clearly not mapping to that locus. Furthermore, the significance of these results to a wider population of patients with VUR is unknown. A potential complicating factor in genetic studies of the disorder is the well-established tendency for VUR to regress in the first two decades of life [3]; hence, apparently disease-free or asymptomatic relatives of VUR probands should not be classified as "normal" in association or segregation analyses.

We have recently shown that genetic ablation of *uroplakin III* (*UP III*) caused VUR in mice [10]. Uroplakins (UP) are a group of four membrane proteins that are synthesized as major differentiation products by mammalian urothelium, the lining of most of the lower urinary tract, including proximal urethra, bladder, ureter, and renal pelvis. The four UPs [i.e., UP Ia (27 kD), UP Ib (28 kD), UP II (15 kD), and UP III (47 kD)] are highly conserved in all mammalian species examined so far, including humans [11–16]. These integral membrane proteins form 16 nm particles that are packed hexagonally forming two-dimensional crystals ("urothelial plaques") that cover almost the entire apical surface of superficial urothelial umbrella cells [17–21]. Nearest-neighbor analysis by chemical crosslinking showed that UP Ia and UP Ib, which are 40% identical and belong to the "tetraspanin" supergene family [22–24], are crosslinked selectively to UP II and UP III, respectively, suggesting that the four uroplakins form two pairs (consisting of UP Ia/UP II and UP Ib/UP III [25–27]). Ablation of *UP III* led to major changes in urothelial morphology and function, including the absence of a typical superficial umbrella cell layer; reduction of apical urothelial plaque size; compromised urothelial function as a permeability barrier; and, interestingly, VUR in 50% to 60% of the mice of both genders, sometimes associated with hydronephrosis [10, 28]. These results provide direct proof that UP proteins are subunits of urothelial plaques and that UP III contributes to the formation of urothelial permeability barrier [10, 28].

To determine whether mutations in *UP* genes might play a role in human VUR, we genotyped all four *UP* genes in a population of 76 VUR patients and found 18 single nucleotide polymorphisms (SNPs), seven of them missense. Since symptom-free relatives of the VUR probands are not reliable negative controls for VUR [29], we used 90 race-matched healthy individuals, unrelated to the VUR patients, as controls to study the association of these SNPs with VUR. We found only a weak association between two UP polymorphisms with VUR suggesting that missense changes of UP genes cannot play a dominant role in causing VUR in humans, although they may be weak risk factors contributing to a complex polygenic disease.

METHODS

Subjects

The patients and their family members were assessed by pediatric and adult urologists and/or nephrologists, and the inclusion of patients for studies was approved by the Institutional Review Boards of the various participating groups. Patients who presented to the pediatric urology clinic with a presumptive diagnosis of reflux, as determined by a febrile urinary tract infection (UTI), recurrent infections, or moderate to severe hydronephrosis had a full work-up with a cystogram. Either a fluoroscopic or radionuclide voiding cystogram were performed by infusing radiocontrast or radionuclide material into the bladder through a catheter. Demonstration of the contrast agent or radiotracer material into the ureters and renal pelvis confirmed the diagnosis of reflux. VUR had been diagnosed in all patients, except those included in the Newcastle-upon-Tyne group, using micturating cystourethrograms or, in some girls, by radionucleotide cystography. The patients included in the Newcastle-upon-Tyne group were mainly assessed as adults and had typical appearances of reflux nephropathy on intravenous pyelogram; the characteristics of this group have been described in detail before [30]. Patients who had evidence of secondary causes of VUR (e.g., structural bladder outflow obstruction) or other complicating clinical features (e.g., complex multiorgan syndromes with VUR, such as the renal-coloboma syndrome [29]) were excluded from this study. Of the 76 VUR patients (from Pittsburgh, New York, and London) who we used for detecting polymorphisms of *UP* genes, 59 were probands from the same number of families affected by more than one

member with VUR and 17 were apparently sporadic cases; the additional 167 patients (from Boston and Newcastle-upon-Tyne) were all probands of the same number of families. In the association study, the "controls" were race-matched, healthy individuals unrelated to the VUR patients; this is an "unselected" normal population that were not screened and therefore ~1% of them would be expected to have VUR. Informed consent was obtained from individuals before they were included in the study. Total genomic DNA was purified from peripheral blood leukocytes using a blood DNA extraction kit (Qiagen; Valencia, CA, USA).

Genomic structure of the human UP genes

The genomic structure of the coding sequence of human uroplakin genes was determined by aligning cDNA and genomic sequences. A search of the human genome sequence in NCBI database using four human UP cDNA sequences (GenBank accession no. AF085807 for *UP Ia*; AB002155 for *UP Ib*; NM_006760 for *UP II*; and NM_00695 for *UP III*) yielded the following genomic sequences containing uroplakin genes: overlapping cosmids with GenBank accession number AC002115 (*UP Ia*), BAC RP11-484M3 (*UP Ib*), BAC RP11-110I1 and RP11-158I9 (*UP II*), and BAC CTA-268H5 (*UP III*). These genomic sequences were used to determine the exon/intron junctions of the UP genes.

Immunohistochemical staining

Terminated human embryonic tissues at 65 and 70 days' gestation were collected with full ethical approval and were provided by the Wellcome Trust/Medical Research Council Human Embryo Bank held at the Institute of Child Health, London. Bladders were collected from Balb/c mice at different developmental stages. Paraffin sections were deparaffinized, microwaved, and stained using rabbit antisera to total bovine UPs, mouse monoclonal antibodies, or to synthetic peptides of individual UPs [10, 25].

Polymorphism detection

Based on the exon/intron junctions, we designed primers (see **Results** section) to amplify all human UP exons and 50 to 150 bp of their flanking intronic sequences. Polymerase chain reaction (PCR) reactions were performed in a total reaction volume of 30 μL containing 50 mmol/L Tris-HCl (pH 9.1), 16 mmol/L ammonium sulfate, 3.5 mmol/L $MgCl_2$, 4.5 μg bovine serum albumin (BSA), 6 pmol of each primer and 0.2 mmol/L deoxynucleoside triphosphate (dNTP), plus 20 ng genomic DNA and 1 U Taq DNA polymerase. PCR was initiated with a denaturation step of 95°C for 4 minutes, followed by 35 cycles of 20 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C, and a final 10-minute extension at 72°C. PCR products were treated using a presequencing kit (USB, Cleveland, OH), and sequenced using a Big Dye Terminator kit (Applied Biosystem, Foster City, CA, USA). For the specific sequencing primers see Table 2. Sequence variants were identified using the Sequencher 3.1 software (GeneCodes, Ann Arbor, MI, USA).

Table 1. Chromosomal location of uroplakin (UP) genes

	Ia	Ib	II	III
Bovine	18	1	15	5 or 6
Mouse	7	16B5-C2	9	15E2-E3
Human	19q13.1	3q13.3-q21	11q23	22q13.2–13.3

Statistical methods

Chi-square tests (or Fisher exact tests) were used to compare the distributions of genotype frequencies for each of the six polymorphisms of interest (*UP Ia* A7V, S31A, and M255T; *UP Ib* R113Q; *UP III* -73A/G and P154A) individually in the VUR group compared with the control group. Odds ratios for each homozygous genotype relative to the heterozygous genotype were estimated for the case compared with the control group with 95% confidence intervals. These analyses were conducted separately for the first 76 VUR patients and 90 healthy individuals (batch 1) and the additional 167 VUR patients and 60 normal subjects (batch 2). In addition, for the SNP with replication (two batches), the analyses were conducted adjusted for batch using the Mantel-Haenszel test [31]. All *P* values were two-sided. No adjustments were made for multiple comparisons.

RESULTS

Chromosomal location, exon-intron junctions, and expression of human *UP* genes

We have previously mapped the chromosomal location of several *UP* genes in mouse, cattle and human. The cattle genes were mapped through their segregation in a panel of (bovine × rodent) somatic cell hybrids [32]. The mouse genes were mapped by analyzing the inheritance of restriction fragment length variants in recombinant inbred mouse strains, and by fluorescence in situ hybridization (FISH) analysis [32, 33]. Subsequent data from FISH analyses [34] and human genomics assigned human *UP Ia*, *UP Ib*, *UP II*, and *UP III* genes to 19q13.1, 3q13.3-q21, 11q23, and 22q13, respectively (Table 1). Since the genomic sequences of the four major human uroplakin genes are already completed, their intron/exon boundaries were determined by the alignment of the cDNA and genomic sequences (Fig. 1) (Table 2). Studies of mouse and human embryonic bladder tissues by immunohistochemical staining indicated that UP genes were expressed

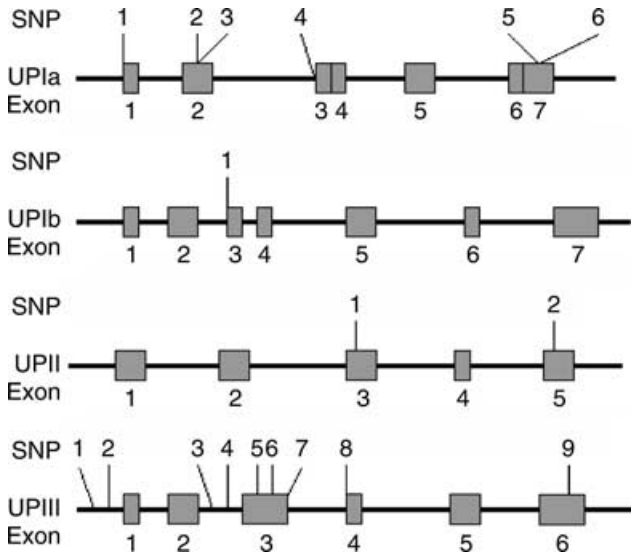

Fig. 1. Genomic structure of human genes encoding uroplakins (UPs) Ia, Ib, II, and III. Boxes and lines denote exons and introns, respectively. The numbers above each gene denote the detected single nucleotide polymorphisms (SNPs). Human *UP Ia*, *UP Ib*, *UP II*, and *UP III* genes span about 11.6, 18.5, 2.2,and 10.9 kb, respectively.

in mouse fetal bladder urothelium as early as day 13 gestation (Fig. 2A to E), and in human fetal bladder urothelium as early as 65 to 70 days gestation (Fig. 2F to I).

Association study of UP SNPs and VUR

We genotyped the four human UP genes in 76 VUR subjects by PCR amplification of the 25 exons (~5.2 kb of amplified exonic sequences per individual) and their 50 to 150 bp of flanking intron sequences (~4.4 kb intronic sequences per individual); see Table 3 for the intronic and other PCR primer sequences). Sequencing of the PCR products (totaling ~10 kb per individual) revealed 18 SNPs (Table 4). Of these, seven polymorphisms in exons resulted in substituted amino acids (Ala7Val, Ser31Ala, Tyr251Phe/Cys, and Met255Thr for *UP Ia*; Arg113Gln for *UP Ib*; and Gln91Leu and Pro154Ala for *UP III*); six other polymorphisms in the exons did not change the amino acid sequence and were thus silent; and five were in noncoding regions (Table 4). Although we could not rule out the possible deletion of an entire allele in samples showing apparent homozygosity, no frame-shift mutation or truncation was detected in any of the VUR samples. In the controls we focused on the genotype of SNPs that cause amino acid substitutions as well as those residing in the 5′-promoter region, because mutations in these regions were more likely to be functionally significant than those that were silent or were in the noncoding introns. SNPs with a minor allele frequency of <5% were not analyzed due to insufficient statistical power. Based on these criteria, we compared the genotype frequency of three SNPs of *UP Ia* (SNPs 1, 2, and 6), one of *UP Ib* (SNP 1), and two of *UP III* (SNPs 1 and 7), in our control and VUR groups (Table 5). The results show that the genotype frequencies of *UP Ia* SNPs 2, 6, *UP Ib* SNP 1, and *UP III* SNP1 of VUR were not significantly different from the control group (Table 5). The only SNPs for which the distributions of genotypes were marginally different between the control and VUR groups were the SNP 1 of *UP Ia* and SNP 7 of *UP III* (both with $P = 0.08$).

We further analyzed the SNP 7 of *UP III*, which caused a proline-to-alanine change, by PCR-direct sequencing and by PCR-restriction enzyme digestion, using a second batch of additional 60 normal and 167 cases of VUR. The *UP III* SNP 7 was characterized by a C to G conversion leading to the mutation of proline 154, which is highly conserved in mouse, rabbit, pig, bovine and human UP III in 145-DPNF(Q/R)GLCN**P**L(S/T)AATEYRFKYVLV-169. The minor G allele was present in 15.7% of 150 normal individuals but in 23.3% of 243 VUR patients. Although the two batches of samples yielded somewhat different results possibly reflecting the heterogeneity/selection of the patient population (see **Discussion** section), the genotype frequencies of the control group (G/G at 2%, G/C at 28%, and CC at 70%) were significantly different from those of the VUR population (G/G at 6.2%, G/C at 34.2%, and CC at 59.6%) (Table 5; $P = 0.036$ in combined data; adjusted for batch using the Mantel-Haenszel test). While the genotypes GG vs. GC or CC vs. GC were not statistically different in VUR vs. controls, the genotype of GG had 4.2 times the odds of VUR compared with the genotype of CC (95% CI of 1.14, 15.36). These data suggest that the G allele was a weak but significant risk factor for VUR.

DISCUSSION

We have reported earlier that genetic ablation of *UP III* gene in mice results in a number of striking urothelial changes, including the conversion of the superficial cells from squamous (umbrella) to cuboidal or even columnar in shape. Moreover, about 50% of the UP III–deficient mice have vesicoureteral reflux, as established by a simple Indian Ink assay and by voiding cystoureterogram [10], a procedure used for the definitive diagnosis of VUR in human patients. Many of these UP III–deficient mice also have hydronephrosis, which in humans is frequently associated with severe VUR. Similar results were obtained when we recently knocked out another mouse gene that encodes UP II—the single-transmembrane-domained member of the other UP pair consisting of UPII/UPIa (Kong XT, et al, in preparation). These results demonstrate that ablation of UPs II and III can cause VUR in mice [10, 28]. It is important to note that in these mouse studies only homozygotes missing both UP III alleles exhibit VUR [10]. The mechanism by which UP deficiency causes VUR in mice is unclear. It is thought,

Table 2. Intron-exon junctions of the human uroplakin genes

Exon	Exon[a] Size *bp*	Exon/intron junction[b] Acceptor	Exon/intron junction[b] Donor	Intron Size *bp*
UP Ia				
1	79		CATTATTCTG/gtgagactcg	1557
2	201	ctctgtccag/CTGTCAGGCC	GGTCCTCACG/gtgagactcc	4577
3	75	gtctccccag/TACCTGGTGC	CCGTGACTAC/gtgagcccgg	131
4	108	ttctcctcag/ATGGTGTCCA	CATGATTGAG/gtgggcgggg	2294
5	180	ttcactctag/CAAGAATGCT	GTTCACCAAG/gtgtggccgt	1792
6	84	ccctgcccag/GGCTGCTTCG	GATGTGGACG/gtgagaggcg	79
7	491	tcctcgccag/CTCCCGGTCA		
UP Ib				
1	96	ctttcaacag/TGCCTTCAGC	GATTATTGGT/gtaagtaatg	963
2	201	ttgcccccag/TGTTGCGGCA	TCTTCTGGCG/gtaagacctc	2268
3	75	tcccttccag/TATTTCATTC	ACAAGACTTT/gtgagtacaa	661
4	123	ccttttatag/TTCACACCCA	CATGCTCCAG/gtaagactgt	3113
5	180	ttgctgacag/GACAATTGCT	TCACAATCAG/gtgagtcctc	4657
6	84	actccttcag/GGCTGCTATG	CTGCTGGACT/gtgagtattt	4775
7	1241	tctattccag/TTTTGGGTTC		
UP II				
1	109	ggaaccccag/CCTGCCAGCA	GGGGCTGCAG/gtctcttcca	456
2	132	ctcccatcag/ACTTCAACAT	GACAGCAAAG/gtctgctacc	193
3	139	ttctgcccag/TGGTGACGTC	CCAAATTCTA/gtaggtactg	251
4	71	accacaaaag/CATTTCCTAC	ACACTCCCTC/gtaagtaaca	428
5	463	cttttgacag/GAAGGAACAT		
UP III				
1	79		TTCGGCTCGG/gtaggcggtg	875
2	156	cctcctccag/CTGTGAACCT	GTCGACTCAG/gtaagggtcc	1075
3	280	gcctctgcag/CCATTTCCAG	CGGAGTACAG/gtgggtgtaa	1609
4	83	ctccttccag/GTTCAAGTAT	ACCAACCAGC/gtaagtggtg	4037
5	133	cccaccacag/TCACCCCATA	TCAGCCTCGT/gtaagtacct	2246
6	322	ccttggacag/GGACATGGGG		

All introns follow the GT-AG rule except for intron 5 of uroplakin II.

[a]Based on GenBank accession no. AF085807 (UP Ia), AB002155 (UP Ib), NM_006760 (UP II), and NM_006953 (UP III). The first exon of each gene includes the 5′-UTR and the last exon of each gene includes the 3′-UTR.

[b]Exon sequences are capitalized and intron sequences are in small letters.

however, that since UP expression begins quite early in urothelium covering the entire lower urinary tract during embryonic development (Fig. 2), UP deficiency may lead to widespread urothelial abnormalities that in turn affect the development of lower urinary tract leading to VUR, possibly due to a lateral displacement of the vesicoureteral junction [35]. Additional experiments are being conducted to determine whether this mechanism indeed operates in the UP-deficient mice.

To test for the possible involvement of UP defects in VUR in human patients, we have analyzed the entire coding sequences as well as 50 to 150 bp of their flanking noncoding/intronic sequences of the four major UP genes from 76 VUR patients and found 18 SNPs (Table 4). Further analysis of five of them that are missense and have a minor frequency of >5%, as well as an SNP in the 5′-nontranscribed region of *UP III* gene, showed that only two of them, the Pro154Ala of UP III and the Ala7Val of UP Ia, were weakly associated with VUR (Table 5). Additional screening of the UP III SNP using another batch of VUR patients and normal controls confirmed a weak association (Table 5). The main conclusion from these results is that some UP alterations, such as the ones we identified here, may play a role, albeit a relatively minor one, in VUR (see below). Specifically, the proline to alanine change in UP III could lead to the loss of a "proline-kink" thus resulting in a major change in protein conformation and possibly protein stability [36–38]. Although these results indicate that UP alterations can play a minor role in VUR, perhaps the most significant and somewhat surprising conclusion from our data is that major UP mutations are not found in patients with primary VUR.

Given the striking VUR and severe hydronephrosis phenotype seen consistently in about 50% of the UP III–deficient mice [10], and more recently, in the UP II–knockout mice (unpublished observations), we anticipated that UP defects might play a prominent role in human VUR patients [10, 39]. Our results indicate, however, that genetic changes in UP genes are, at best, involved in a minor manner in the population of VUR patients that we have studied (Table 5). There are several possible explanations for this apparent contradiction between the mouse and human data. First, human UP genes may be functionally redundant. This seems unlikely, however, given the fact that the amino acid sequences of UPs are highly conserved [11, 15], and that genetic ablation of UP III and II genes yielded striking phenotypes

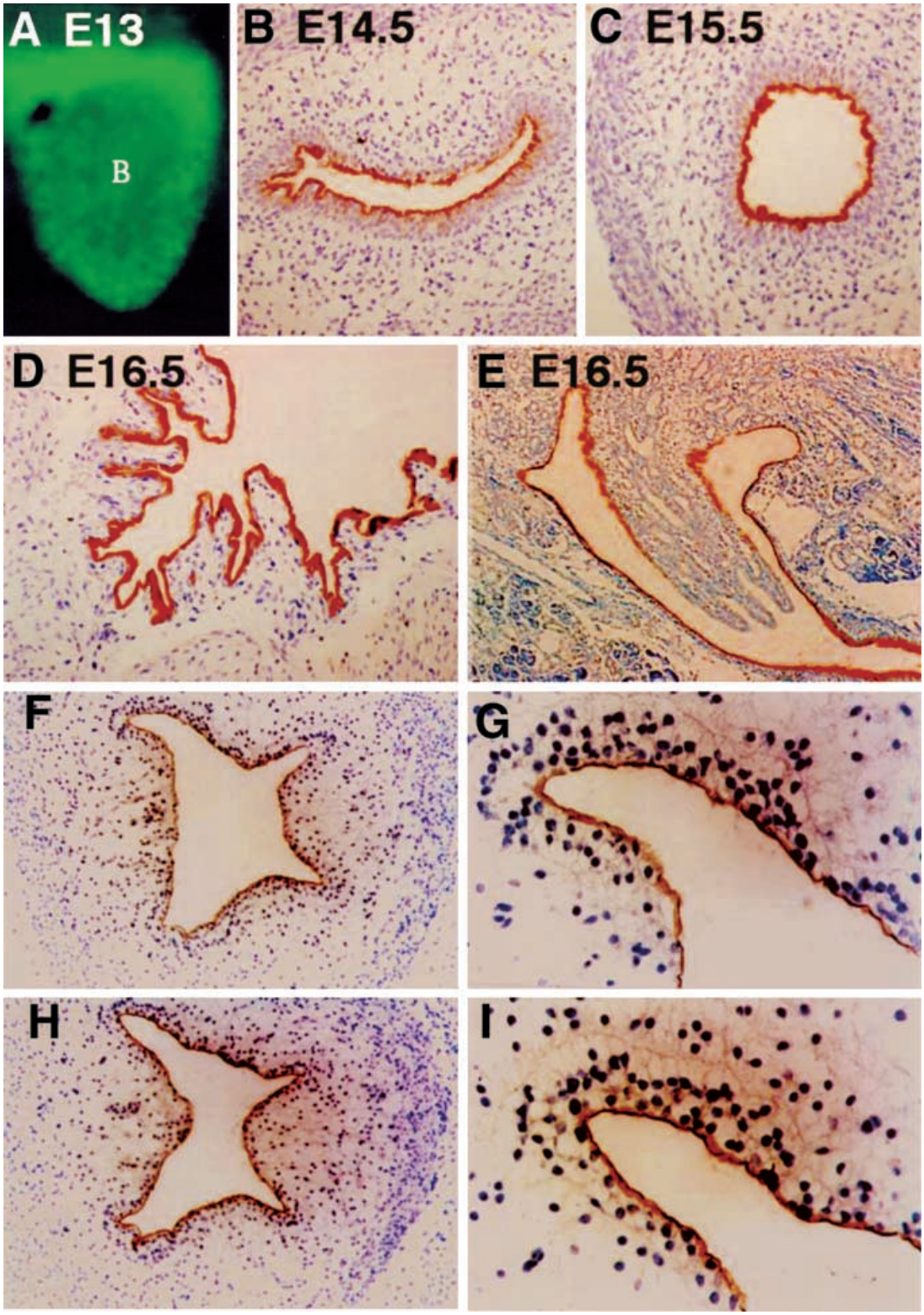

Fig. 2. Expression of uroplakin (UP) in embryonic mouse (*A* to *E*) and human (*F* to *I*) urothelium. Whole-mount (A) or paraffin sections of mouse bladder of E13 (A), E14.5 (B), E15.5 (C), and E16.5 (D), and renal pelvis of E16.5 embryos (E) were stained by immunofluorescence (A) and peroxidase (B to E). A rabbit polyclonal antiserum against total bovine UPs (A) or a mouse monoclonal antibody to UP III (B to E) was used. Note the strong UP staining of the apical urothelial surface not only in the bladder (A to D), but also in ureter and renal pelvis (E). Human fetal bladder at 70 days' gestation was paraffin-sectioned and stained using a rabbit antiserum to total bovine UPs (F and G) and a rabbit antiserum against UP Ib (H and I). (G and I) The higher magnification views of (F and H), respectively. All sections, except the whole mount in (A), were counterstained with hematoxylin. Note the strong immunostaining of the luminal surface of the fetal human bladder urothelium.

Table 3. Primers used for amplifying the exons and flanking intron regions of human uroplakin (UP) genes

Exon	Size *bp*	Forward primer (5′→3′)	Reverse primer (5′→3′)
UP Ia			
1	450	GGAGTCTGGGTATGGCATGAG[a]	TGAGTCTGCCCGAGAGACAGT
2	426	GGTCTTTGCCCACTTTACTGA[a]	GGAACTCACACTAGTGGCTGG
3	287	AAACTTGCATTTCCCCAGTG	GGAGAACAGGGAAGAACAAGG[a]
4	271	CGGAGGTCGTCCTCTCTCC	GAGATCTCATGAGCGGATGTC[a]
5	323	CAGCTGTGTCAAAGGCCAATCCTG[a]	GATGGATAGACAGATGGAGCAGGGC
6, 7	438	CTCAGGGAAGGTGGTGACTTT[a]	AGTGGAAGGACGTAAGGGCTA
UP Ib			
1	473	AGTCCACCACTCAGAAGGATG	AGGAAGCCAGTAATCACAGCAT[a]
2	480	CTGGCAATGGGGAGTTTAGA[a]	CATGTGGACCATTTCACTGC
3	320	CTAGCACCAAGCCTCAATCC	TGCCTGGGCACATAGTAAGG[a]
4	363	CGCCTCTGAGTACAGTTTTGC[a]	AAGCTAATCATCATGGCTTGAA
5	405	TGGCTCTAGTAGGGACCACG[a]	TCCTGGTTTAGCATTTGCAG
6	230	AACCAGTGGCACTCTCAAGG	CCTGCTTTCCCATCAATCTC[a]
7	314	GCCTGAATGAACCTAACATCCAC[a]	CATGAGAACTGCAGAAGCCTG
UP II			
1	466	CAGGAACACTGCCTTATGCACG	AGACCTCTCTTTGAGAGCCCTGG[a]
2	310	CTGGTGGTCATACTGGCACA[a]	GGGACTAGAGGGATGCCTTGG
3	262	CCTGGGGTACCCACACTCTA[a]	CTTCCCTAGGTGCCTCAGGCT
4	277	GCCCCTCTTCCTGTAAGYC[a]	GGTTCCGTGCTAGGATAGAGG
5	286	GTGGTCTCCCCTCTCTTTTG	AGAGGAGGGCAGGAGGAG[a]
UP III			
1	839	GAACCGAAGGGGTCATACTAGGCGT[a]	CTGGGGCTAGTCCCTTCTCTGTCCT[a]
2	344	CACGTGCTCAGTAACTGCAAG[a]	ATGAGACGACTACAGGCATGG
3	456	AGGATGATCAGGCATTTGATG	CAAGAAGTGTGGCTGCTCCTA[a]
4	217	ACCCCTAGGCCATCCTACATCCC	CCTTCTCGGGTCCCCTTGAGCATC[a]
5	258	GTGCGGTGTCTGGGAAGTAAC	GAGGACCCCACTGCTGAGTC[a]
6	349	AAGGCATTTGGGTTTGCTCT[a]	AAGGGTTTTCCTGCAGTTGGA

[a]Primers used for DNA sequencing reactions.

Table 4. Variations and allelic frequency of the human uroplakin (UP) genes from 76 vesicoureteral reflux (VUR) patients

					Frequency %	
SNP	Location	Position[a]	Common	Variant	VUR	control
UP Ia						
1	Exon 1	21	G<u>C</u>C (Ala^{7})	G<u>T</u>C (Val)	10.5	15.6
2	Exon 2	92	<u>T</u>CC (Ser^{31})	<u>G</u>CC (Ala)	9.9	11.7
3	Exon 2	94	TC<u>C</u> (Ser^{31})	TC<u>T</u> (Ser)	0.7	
4	Exon 3-5′ acceptor	[−26]	G	T	8.7	
5	Exon 7	753	T<u>A</u>T (Tyr^{251})	T<u>T</u>T (Phe)	1.5	
	T<u>A</u>T (Tyr^{251})	T<u>G</u>T (Cys)	1.5			
6	Exon 7	765	A<u>T</u>G (Met^{255})	A<u>C</u>G (Thr)	42.8	46.7
UP Ib						
1	Exon 3	345	C<u>G</u>A (Arg^{113})	C<u>A</u>A (Gln)	6.6	6.7
UP II						
1	Exon 3	254	AC<u>G</u> (Thr^{72})	AC<u>A</u> (Thr)	0.8	
2	Exon 5	592	T<u>A</u>A (Stop)	T<u>G</u>A (Stop)	14.8	
UP III						
1	5′ UTR	[−73]	G	A	25.7	17.2
2	5′ UTR	[−25]	G	C	2.9	
3	Exon 2-3′ donor	84	C	A	1.4	
4	Exon 3-5′ acceptor	[−17]	C	G	5.1	
5	Exon 3	272	C<u>A</u>A (Gln^{91})	C<u>T</u>A (Leu)	0.7	
6	Exon 3	402	GT<u>C</u> (Val^{134})	GT<u>T</u> (Val)	22.4	
7	Exon 3	460	<u>C</u>CA (Pro^{154})	<u>G</u>CA (Ala)	25.0	15.6
8	Exon 4	549	TC<u>A</u> (Ser^{183})	TC<u>G</u> (Ser)	35.9	
9	Exon 6	858	CA<u>A</u> (Gln^{286})	CA<u>G</u> (Gln)	43.5	

VUR is vesicoureteral reflux. The number of single nucleotide polymorphisms (SNPs) detected in UP genes varied significantly. UP Ib and UP II genes contained only one and twp SNPs, respectively, while UP Ia and III genes contained six and nine SNPs, respectively. Thus, the SNP density ranged from one SNP per 2.2 kb for Ib to one per 233 bp for III, with an average of one SNP per ∼410 bp. Twelve of the 18 SNPs resided in the coding regions, with only six in the noncoding regions; thus the SNP density of the coding regions was 3.5-fold higher than that of the noncoding regions.

[a]Based on GenBank accession nos. AF085807 (UP Ia), AB002155 (UP Ib), NM_006760 (UP II), and AB010637 (UP III).

Table 5. Case-controlled analysis of the association between vesicoureteral reflux (VUR) and uroplakin (UP) polymorphisms

Single nucleotide polymorphism	Genotype	Vesicoureteral reflux *number* (%)	Control *number* (%)	*P* value[a]	Odds ratio	95% CI limits
UP Ia 1	CC	63 (82.9)	64 (71.1)	0.08	CC vs. CT 2.38	1.69, 5.55
(Ala7Val C->T)	CT	10 (13.1)	24 (26.7)		CC vs. TT 0.28	0.04, 1.9
	TT	3 (4)	2 (2.2)		TT vs. CT 3.6	0.52, 24.93
UP Ia 2	GG	4 (5.3)	2 (2.2)	0.13	GG vs. GT 4.76	0.72, 33.3
(Ser31Ala T->G)	GT	7 (9.2)	17 (18.9)		GG vs. TT 2.17	0.38, 12.5
	TT	65 (85.5)	71 (78.9)		TT vs. GT 2.22	0.87, 5.7
UP Ia 6	CC	17 (22.4)	19 (21.1)	0.36	CC vs. CT 1.33	0.59, 2.94
(Met255Thr T->C)	CT	32 (40.8)	46 (51.1)		CC vs. TT 0.8	0.34, 1.88
	TT	28 (36.8)	25 (27.8)		TT vs. CT 1.66	0.86, 3.37
UP Ib 1	AA	0 (0)	0 (0)	0.97		
(Arg113Gln G->A)	AG	10 (13.2)	12 (13.3)			
	GG	66 (86.8)	78 (86.7)		GG vs. AG 1.01	0.41, 2.5
UP III 1	AA	6 (7.9)	3 (3.3)	0.19	AA vs. AG 1.85	0.42, 8.33
(−73 G->A)	AG	27 (35.5)	25 (27.8)		AA vs. GG 2.8	0.68, 12.5
	GG	43 (56.6)	62 (68.9)		GG vs. AG 0.64	0.33, 1.25
UP III 7	GG	7 (9.2)	2 (2.2)	0.08	GG vs. GC 3.5	0.65, 18.1
(Pro154Ala C->G)	GC	24 (31.6)	24 (26.7)		GG vs. CC 4.98	0.98, 25.08
	CC	45 (59.2)	64 (71.1)		CC vs. GC 0.7	0.36, 1.39
UP III 7 (batch 2)	GG	8 (4.8)	1 (1.7)	0.37	GG vs. GC 2.44	0.28, 20.84
	GC	59 (35.3)	18 (30)		GG vs. CC 3.28	0.39, 27.1
	CC	100 (59.9)	41 (68.3)		CC vs. GC 0.74	0.39, 1.4
UP III 7	GG	15 (6.2)	3 (2)	0.036[b]	GG vs. GC 3.02	0.8, 11.4
(batch 1 and batch 2[b])	GC	83 (34.2)	42 (28)		GG vs. CC 4.2	1.14, 15.36
	CC	145 (59.6)	105 (70)		CC vs. GC 0.73	0.45, 1.16

[a]Chi-square test for association; [b]Mantel-Haenszel chi-square test for general association.

including VUR and hydronephrosis [10, 28]. Second, only one healthy allele of UP gene may be functionally adequate. This may be particularly true if the mutation makes the UP less efficient in interacting with other UPs; if so the UP encoded by the remaining "healthy" allele would be functional while the mutated UP would be excluded from interacting with other UPs and thus would be in effect silent. This may be the case for the Pro154Ala mutation of *UP III*, which is expected to introduce a major change in protein conformation [36, 37]. Third, the altered UP conformation may be "corrected" and thus tolerated once the mutant protein is incorporated into the presumably tightly packed UP complex [21, 40]. In this regard, it is known that the extracellular domains of UPs interact with one another tightly in forming the 16 nm particle, such that many of the antigenic epitopes defined by synthetic peptides become inaccessible in intact urothelial plaques [25]. Fourth, we have found that, although some of the breeding pairs that we used to generate the UP III−/− homozygote offspring yielded litters that can survive into adulthood despite their VUR and associated hydronephrosis, some other breeding pairs yielded offspring that consistently die around days 8 to 10 postnatally, with elevated blood urea nitrogen suggesting renal failure [10]. In additional, we recently found that some of the breeding pairs of UP II knockout mice also yielded litters that underwent similar VUR, renal abnormalities, and neonatal death (unpublished). These results suggest that a major UP defect, when combined with a certain genetic background, can be neonatally lethal in mice. In this regard it is important to note that, in humans, urinary tract malformations are among the most common of all congenital anomalies, and are principal reasons for both medically indicated terminations of pregnancy and also can cause fetal, perinatal, and infant death [41, 42]. This raises the interesting possibility that major human UP defects may also cause renal and other abnormalities resulting in prenatal or postnatally lethality; such a possibility could explain why we have not encountered major UP defects, including truncation and frame-shift, in our VUR analysis. Fifth, although our data cannot rule out the possibility that causative mutations in the intronic sequences that are destroyed in the knockout mice are responsible for the observed mouse phenotype, our current data suggest that missense changes of UP genes cannot play a dominant role in causing VUR in human since we found only a weak association between UP mutations and VUR phenotype, a lack of significant mutations in the 50 to 150 bp flanking introns, and, importantly, a lack of families with simple dominant Mendelian inheritance (unpublished observation). Additional studies are needed, however, to address whether mutations in 5′- and/or 3′-noncoding genomic sequences, as well as intronic sequences that are not covered in our current study but can potentially cause diseases [43], play a contributing role in VUR. Finally, and perhaps most important, our data are consistent with the hypothesis that VUR is a complex and heterogeneous symptom that can be caused

by a large number of genetic defects that affect multiple cell types in the lower urinary tract. Nishimura et al [44] showed that a large portion of mice lacking the angiotensin type 2 receptor (AT2R) gene exhibited VUR while a smaller percentage also exhibited other congenital anomalies, including megaureter, ureteral duplication, and multicystic dysplastic kidneys [44]. However, a small scale survey of 23 VUR patients suggest that defects in the AT2R gene may not be involved in VUR [45]. This situation is therefore analogous to ours and suggests that mutations in AT2R and UPs may be involved in only small subpopulations of human VUR patients. The heterogeneity of human VUR is strongly supported by Feather et al [9] who studied, by genome-wide search, seven European families whose members exhibit apparently dominant inheritance of VUR. This study identified in one of the pedigree a positive locus on chromosome 1p13, which clearly could not be related to the four major UP genes (Table 1) and was not involved in other pedigrees [9].

Overall, our results are consistent with the idea that VUR is a heterogeneous disease that is caused by defects in a panel of genes that are expressed in the lower urinary tract. Knockout mice lacking such genes provide well-defined and valuable experimental models for studying certain aspects of VUR. However, statistical analysis of a general population of VUR patients may mask the involvement of individual genes in such a heterogeneous disease. Genome-wide search using large pedigrees of VUR [9], coupled with association studies [46], is needed to pinpoint genetic defects, which could include those of UP and AT2R genes, that underlie the VUR phenotype within specific pedigrees.

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