Mutation analyses of *Uroplakin II* in children with renal tract malformations

Dagan Jenkins¹, Maria Bitner-Glindzicz¹, Sue Malcolm¹, Jennifer Allison¹, Rose de Bruyn¹, Sarah Flanagan², David F. M. Thomas³, Rachel A. Belk³, Sally A. Feather³, Coralie Bingham², Jennifer Southgate⁴ and Adrian S. Woolf¹

¹UCL Institute of Child Health, and Great Ormond Street Hospital NHS Trust, London, ²Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter, ³St James’ University Hospital, Leeds and ⁴Jack Birch Unit of Molecular Carcinogenesis Department of Biology, University of York, York, UK

**Abstract**

**Background.** Uroplakin (UP) proteins cover urothelial apical surfaces. Mice lacking UPIIIa have elevated urothelial permeability and congenital renal tract anomalies, and *UPIIIa* mutations have been reported in children with kidney and ureter malformations. Mice with null mutation of another UP family member, *UPII*, are often born with congenital hydronephrosis. We hypothesized that *UPII* mutations may be present in humans with renal tract malformations.

**Methods.** Mutations were sought, using direct sequencing of the five *UPII* exons, in 42 children with diverse renal tract anomalies.

**Results.** No *UPII* abnormalities were detected in 41 patients, whereas one index case had a heterozygous frameshift change which, if expressed, would generate a truncated protein. This Caucasian child presented with vesicoureteric reflux (VUR), bilateral nephropathy and renal failure. The genetic change was also found in the index case’s mother who had normal renal ultrasonography, but it was absent in 150 healthy Caucasian control individuals (96 assessed by direct sequencing and another 54 assessed by restriction digests). *UPII* was immunolocalized in urothelium of the normal human embryonic renal pelvis in a pattern similar to UPIIIa.

**Conclusion.** This study offers no definitive support for *UPII* mutations causing human renal tract malformations. In rare patients, *UPII* variants might be implicated in pathogenesis when acting in conjunction with other yet-to-be-defined, genetic or environmental modifying factors.

**Keywords:** gene; malformation; renal failure; uroplakin; VUR

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**Introduction**

Uroplakin (UP) protein heterodimers (UPIa/UPII, UPIb/UPIIIa) form multiple thickened plaques of ‘asymmetric unit membrane’ located on the apical surface of the urothelium, the specialized epithelium that lines the renal tract from the pelvis to the proximal urethra [1,2]. In mammals, the full complement of UPS have so far only been reported to be expressed in the renal tract [3], although UPIb itself has been detected in corneal [4] and respiratory [5] epithelia, and, in *Xenopus*, the homologous UPIII protein is detected in lipid rafts on the surface of unfertilized eggs [6].

UPIIIa contributes to the ‘water-tight’ properties of urothelia [7], and this protein is also essential for normal renal tract development because mice which lack both alleles of *UPIIIa* are born with severe vesicoureteric reflux (VUR) [8]. Subsequently, *de novo* heterozygous mutations in *UPIIIa* were reported in four children with kidney failure caused by renal aplasia and/or dysplasia [9]. The discovery of human *de novo* mutations has implications for genetic counselling because, now that children with end-stage renal failure are increasingly surviving to reproductive age [10], future generations may be at increased risk of renal malformations. UPIIIa is normally expressed in embryonic urothelia but not in the developing kidney itself [9,11], leading Jenkins *et al.* [9] to hypothesize that a primary perturbation in urothelial differentiation can lead to human kidney malformations, perhaps by generating a functional impairment of fetal urine flow. A similar mechanism had been demonstrated in mouse models with mutations of other genes including *calcineurin B* type 1 and *sonic hedgehog* [12].

Mice with null mutation of another member of the UP family, *UPII*, lack urothelial plaques and often have congenital hydronephrosis associated with either primary VUR or with urothelial cell overgrowth,
the latter causing ureteric obstruction [13]. These observations led to the hypothesis, tested in the current study, that UPII mutations may be present in humans with renal tract malformations.

Patients and methods

Patients

We studied 42 children with diverse renal tract anomalies, ascertained by the Nephrology Department at Great Ormond Street Hospital, London [9] and the Urology Department at St James’s University Hospital, Leeds, UK [14]. The same individuals were described by Jenkins et al. [9] in a study which sought mutations of UPIb and UPIIIa. In 17 of the children, the primary diagnosis was bilateral renal aplasia or dysplasia including several cases in which primary VUR was associated with bilateral nephropathy: none of this group had overt urinary tract obstruction. As detailed in this report, we found a UPII insertion in one female subject from this latter group and her detailed clinical history is given in the ‘Results’ section. In six other children (all boys), the primary diagnosis was posterior urethral valves, a congenital lesion resulting in obstruction of bladder outflow; mostly, this was associated with bilateral nephropathy. The other 19 children had multicystic dysplastic kidney and their detailed clinical features have been previously published [14]. None had clinical evidence of a multiorgan syndrome associated with renal tract malformations such as the renal-coloboma or branchio-oto-renal syndromes [15].

Sequencing of genomic DNA

After parental consent, venous blood was collected from patients and first-degree relatives; leucocyte DNA was extracted by the salt-precipitation method. The exonic structure and polymorphic variation of UPII used as a reference for mutation analysis was that defined by Jiang et al. [11] (Ensembl accession number: ENSG00000110375). All five exons of UPII were sequenced in all index cases using previously published primers [11] using the same techniques as applied in our previous study of UPIb and UPIIIa [9]. Sequences were analysed using Sequencher v4.1 software. Sequencing was performed using both forward and reverse primers, and the mutation was validated by sequencing of several replicates (i.e. independent PCR products) in the index case and her parents. A restriction digest was also used to confirm the mutation: following amplification of exon 2, the DNA was digested with MsII, giving wild-type products of size 244 and 66 bp, or a 314 bp product from the mutant allele. The mutation was excluded in 96 control individuals (192 chromosomes) by direct sequencing of UPII exon 2 and a further 54 controls were genotyped by the MsII restriction digest and the mutation not detected. In addition, the coding region of hepatocyte nuclear factor 1β (HNF1β) was sequenced as described [16]: mutations of this gene, expressed in the developing renal tract can cause a range of malformations including dysplastic and cystic kidneys, as well as uterus anomalies.

UP immunohistochemistry

Paraffin wax-embedded sections of a normal 7-week gestation human embryo were provided by the Human Embryo Bank held at the UCL Institute of Child Health, London, UK. At this gestational age, the human kidney has only one layer of glomeruli and the pelvicalyceal system is beginning to form by remodelling of the first sets of ureteric bud branches [15]. Dewaxed, rehydrated sections were labelled with titrated monoclonal antibodies against UPS. The UPII monoclonal antibody was produced from a cloned hybridoma cell line, JBU14.7, prepared by fusion of the NSO plasmacytoma cell line with splenocytes from BalbC mice immunized with the peptide sequence GTATESREIPMSTLPRC, which is specific to the extracellular domain of UPII (Pettit et al., manuscript in preparation). The UPIIIa monoclonal antibody (1/50, clone AU1; Progen Biotechnik, Heidelberg, Germany) was raised in mouse by immunization with total bovine UPS and screening hybridoma clones by ELISA for those secreting UP antibodies: on western blots of total urothelial proteins, it specifically reacts with UPIIIa [17]. Endogeneous peroxidase activity was inactivated in peroxidase block (DakoCytomation Ltd, Ely, UK) for 30 min, followed by blocking of endogeneous avidin-binding sites using an avidin/biotin kit (Vector Laboratories, Peterborough, UK) according to the manufacturer’s protocol. Prior to UPIIIa labelling only, sections were subjected to antigen retrieval by boiling in 2.1 g/l citric acid for 6 min. Sections were incubated sequentially in primary antibody, biotinylated rabbit antimouse IgG and strepavidin/HRP ABC complex according to the supplier’s instructions (DakoCytomation Ltd) with washing between each step. Negative controls were performed using purified IgG or omission of primary antibody. Antibody binding was detected using diaminobenzidine as the substrate and slides were counterstained with haematoxylin, dehydrated and mounted in DPX (Sigma-Aldrich Chemical Company, Poole, Dorset, UK).

Results

Clinical case

In the whole group of 42 children with renal tract anomalies, all but one had normal UPII sequencing. This Caucasian female, aged 8 years, presented with a 1-year history of bacteriologically proven urinary tract infections. She also reported nocturia (x1/night) for several years. A micturating cysstourethrogram showed grade IV VUR bilaterally (Figure 1A). Ultrasonography revealed dilated distal ureters bilaterally (Figure 1B), both kidneys being ‘small’ and hydrenephrotic (Figure 1C and D). A 99mTc Technetium-diethylenetriaminepentaacetic acid scan showed poor uptake bilaterally with 71% of the total function on the left. Plasma creatinine was 227 μM and creatinine clearance was 22 ml/min/1.73 m². She was treated with 1α vitamin D for hyperparathyroidism and received microbial prophylaxis with Trimethoprim.

She had been born at 36-week gestation following an uneventful pregnancy, apart from the fact that the
mother had chicken pox when the patient was born: the baby was treated with immunoglobulin and had mild disease. We could not access original obstetric notes but there was no history of antenatally detected renal anomalies. The parents and her two sisters (aged 15 and 22 years) underwent renal ultrasonography with normal findings: both the mother’s parents were deceased at this point (Figure 2A). There was no definite family history of renal disease, although the father had a ‘weak bladder’ and the mother’s sister had hypertension.

One year after presentation, the subject complained of headaches and was found to be hypertensive with left ventricular hypertrophy. Her height (123 cm) and weight (22 kg) were both below the 10th percentile for age and sex. She was in renal failure (plasma creatinine 255 µM) and was acidotic (plasma bicarbonate 16 mM) with a mildly raised parathyroid hormone level. She was treated with nifedipine, hydralazine, frusemide, sodium bicarbonate, vitamin D and calcium carbonate. A midstream urine showed plentiful white cells but without growth and she had proteinuria (3+ on dip-stick). A ⁹⁹ᵐTc-dimercaptosuccinic acid scan showed poor uptake bilaterally with 94% of the total uptake on the left. Ultrasonography showed two bright kidneys with loss of corticomedullary differentiation (right 47 mm long and left 81 mm long; median for age 90 mm); the bladder contained 638 ml, with a post-micturition residue of 107 ml. She underwent a trial of continuous urinary catheter drainage, but her plasma creatinine level failed to fall over 1 week. Subsequently, renal failure progressed, leading to dialysis and cadaveric renal transplantation at 11 years. After rejection episodes and post-transplant lymphoproliferative disease, dialysis was recommenced. Visualization of her optic discs and retina (performed during investigations for headaches) revealed no abnormality. A second cadaveric renal transplant performed at 13 years developed chronic allograft nephropathy, necessitating return to dialysis. At 15 years she had failed to undergo menarche and an ultrasound showed a bicornuate uterus: menstruation finally started at 17 years.

Molecular analyses

A heterozygous genetic change was found in UPII in this patient. Four bases (CACT) were inserted at nucleotide position 215 (Figure 2B). This results in a frameshift predicted to affect the UPII protein at codon 60, altering the seven amino acid residues encoded by codons 61–67 and inserting a premature termination codon at 68; this change was, therefore, designated L60fsX68. The final postulated effect on the UPII protein is shown in Figure 2C and D, i.e. the UPII pre- and pro-sequences fused to a short, novel peptide. The same genetic change was also detected in leucocyte DNA from the mother, but not in the father, both of whom had normal renal ultrasounds.

Fig. 1. Radiology of index patient: (A) Bilateral VUR on micturating cystogram. (B–D) Ultrasonography showed, (B) dilated ureters (⋆) behind bladder (dotted line), (C) and (D) are hydronephrotic right and left kidneys, respectively.
The renal tracts of the two sisters of the index case were also deemed normal as assessed by ultrasound scanning, although DNA samples from them were unavailable for analysis because permission for venesection was not granted; DNA was also unavailable for the two maternal grandparents because they were deceased. This genetic change was absent in 96 Caucasian control individuals (192 chromosomes), as assessed by direct sequencing of UPII exon 2, and an additional 54 controls (108 chromosomes) were also genotyped as homozygous wild-type by MsI restriction digest. The index case was previously shown to be negative for mutations in both UPIb and UPIIIa [9], although she was heterozygous for the non-synonymous SNP at amino acid residue 154 of UPIIIa (Pro154Ala) which Jiang et al. [11] found to be weakly associated with primary VUR. Sequencing of the HNF1b coding region revealed no mutations. Notably, the normal appearance of the index case’s optic discs as ascertained by an expert ophthalmologist would make the renal-coloboma syndrome [18] most unlikely.

**UP immunohistochemistry**

A low power view of the 7-week normal human embryonic kidney is shown in Figure 3A. Immunohistochemistry using specific monoclonal antibodies raised against human UPII (Figure 3B) and UPIIIa (Figure 3C) detected both proteins in the urothelium of the renal pelvis: no signal was detected in a negative control (Figure 3D). UPII immunolocalized predominantly to the apical surface of the urothelium, although fainter signal was also detected apparently on other urothelial cell surfaces: in contrast, UPIIIa was exclusively detected in an apical location.

**Discussion**

This study, the first to examine UPII in human congenital renal diseases, offers no definitive support for UPII mutations causing these conditions. In particular, no de novo mutations were detected in 42 index cases. We did, however, immunolocalize UPII in the urothelium of the developing normal human renal tract in a pattern similar to UPIIIa and we also found a heterozygous frameshift change of UPII in a single index case who had VUR, nephropathy and renal failure. We postulate that UPII mutations may occasionally predispose individuals to renal tract malformations in conjunction with other yet-to-be-defined, genetic or environmental modifying factors.
Following their isolation [19,20], the UPs were generally considered to ‘simply’ confer important physical properties to the asymmetric unit membrane, decreasing its permeability to urine. Indeed, this function was elegantly confirmed by demonstrating that the genetic ablation of \( UPIIIa \) in mice elevates urothelial permeability [7]. However, the observation that \( UPIIIa \) null mutant mice also have congenital VUR and hydronephrosis [8] provided the first indication that the UP family might have additional roles in the differentiation of the renal tract itself. Four humans with renal aplasia/dysplasia and two with VUR were subsequently found to have \( \text{de novo} \) \( UPIIIa \) mutations [9] and another \( \text{de novo} \) change was recently reported in a patient with multicystic dysplastic kidney [21].

The recent observation that VUR and hydronephrosis can be caused by \( UPII \) null-mutations in mice [13] prompted us to seek \( UPII \) mutations in 42 index cases with a variety of renal tract malformations. While the main conclusion to the genetic analyses in the current study must be that \( UPII \) is not a major player in human congenital renal tract disorders, we did note a \( UPII \) insertion in the index case. Her clinical history and investigations were compatible with ‘reflux nephropathy’ associated with VUR. While the kidney damage may have in part been caused by bacterial pyelonephritis, it was notable that urosepsis had only been proven in the year before presentation, when a history of nocturia considerably predated this and there was already marked somatic growth impairment at presentation: this is consistent with a long history of established renal failure. Perhaps her nephropathy had a congenital basis (hypoplasia/ dysplasia), as has been increasingly recognized in the primary VUR population with renal failure [22]. It is also notable that this patient had a bicornuate uterus. While uterine malformations have not been reported in mice null-mutant for either \( UPII \) or \( UPIIIa \) [8,13], a girl with a \( UPIIIa \) missense mutation had persistent cloaca [9], and the evaginating dorsal wall of the human urogenital sinus, destined to form part of the female reproductive tract, expresses UPs [23].

Could the frameshift change found in \( UPII \) have contributed to the pathogenesis of renal disease in the current index case? Here we examine evidence ‘for’ and ‘against’ this contention. Several observations would support a role in pathogenesis. First, it was absent in our panel of 150 healthy Caucasian control individuals (96 assessed by direct sequencing and another 54 assessed by restriction digests), making it rather unlikely that it was a ‘chance occurrence’, unrelated to the renal disease. Second, the genetic change would have major functional implications with regard to the \( UPII \) protein. The frameshift might encode a transcript that undergoes accelerated decay or, if such a transcript were to be translated, it would lead to a radical truncation of the wild-type sequence to generate a protein lacking relevant domains for interaction of pro-\( UPII \) with its plaque partner, \( UPIa \), a process essential for exit of the complex from the endoplasmic reticulum, insertion of \( UPII \) into cell membranes, would also be prevented [2,24] (Figure 2C–E). One can postulate that, in such
a case, the human renal tract malformation in the current individual results from ‘haploinsufficiency’ because the genetic change only affects one of the two UPII alleles. On the other hand, because the glycosylated pro-sequence may regulate formation of UP heterotetramers [2], it is conceivable that the frameshift mutation is ‘dominant-negative’ interfering with the function of UPII protein generated by the wild-type allele. Third, as demonstrated for the first time in this study, UPII protein, like UPIIIa, is immunolocalized to the nascent human urothelium at 7 week gestation; therefore, it could begin to have a critical function at a very early stage of human renal tract development.

Other observations, however, make us question whether the insertion does contribute to pathogenesis. First, an insertion–deletion polymorphism of UPII has been reported in dbSNP (www.ncbi.nlm.nih.gov/SNP; rs5795154) and this would cause a frameshift at amino acid residue 56 leading to a premature termination at residue 60, thus generating a truncated protein similar to the L60fsX68 protein. However, this database entry is unvalidated and this ‘indel’ was not observed by direct sequencing in either 96 controls (this study) or in 76 patients with primary VUR [11]; also, no clinical information is available regarding the individual in whom this variant was apparently detected. Second, our index case’s mother had a normal renal ultrasonography but carried the same heterozygous UPII change like her affected daughter. In this regard, it should be noted that ultrasonography will not detect all structural defects and, in the mother’s case, it was not considered clinically justified to perform cystography (to seek VUR) or intravenous urography/isotope scanning (to seek renal parenchymal/calyceal defects). It could also be postulated that this family exhibits ‘incomplete penetrance’ for renal tract anomalies. In this regard, inheritance in some humans with non-syndromic renal anomalies has been considered autosomal dominant with incomplete penetrance with carriers having only a 50–90% chance of displaying a phenotype [25]. Furthermore, in several human malformation syndromes, the occurrence and severity of renal anomalies varies strikingly between individuals carrying the same mutations, e.g. in the renal-cysts and diabetes (HNF1β mutations; 16) and the renal-coloboma (paired box 2 mutations; 18) syndromes. Additionally, mice carrying UPII null mutations only display hydronephrosis with a penetrance of 80% and the mutation only reproducibly causes juvenile renal failure in the progeny of certain breeding pairs (129/SvEv × Swiss Webster) [13].

Perhaps, rare alleles of UPII encoding a truncated protein are present in the general population and these variants predispose to renal malformation. Congenital renal disease would only result, however, when a heterozygous UPII change was present with other factors which could perturb renal differentiation. Such a factor might be a ‘modifying gene’ and, in this regard, it is noteworthy that the index case was also heterozygous for the Pro154Ala UPIIIa variant significantly associated with primary non-syndromic VUR [11]. It is increasingly evident that diverse environmental factors can alter the trajectory of kidney and urinary tract differentiation [26,27]. As a neonate, the index case was exposed to varicella infection because the mother had chicken pox at the time of delivery. Exposure of fetuses to this virus, especially in mid-gestation, can cause severe neuropathic bladder associated with VUR [28,29]. Even though the current case lacked other classical features of the full-blown congenital varicella syndrome (developmental delay, micophthalmia/-microcornea, limb hypoplasia and gut dysfunction), the large bladder capacity together with incomplete emptying is suggestive of an accompanying neural component. It is possible to postulate that her renal tract disease could have been generated by a (mild) viral infection in an individual who was genetically-predisposed to renal tract anomalies by virtue of carrying a variant of UPII, a gene expressed in the maturing renal tract. In future, it will be of interest to sequence UPII in considerably larger patient groups with renal tract anomalies; such DNA collections are underway e.g. the ESCAPE trial [21] and also the UK VUR DNA Bank (http://www.vur.org.uk/).

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Conflict of interest statement. None declared.

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