Mutations in Uroplakin IIIA Are a Rare Cause of Renal Hypodysplasia in Humans

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Background: Renal hypodysplasia, characterized by a decrease in nephron number, small overall kidney size, and maldeveloped renal tissue, is a leading cause of chronic renal failure in young children. Familial clustering and renal hypodysplasia phenotypes observed in transgenic animal models suggest a genetic contribution. Uroplakin IIIA (encoded by UPIIIA) is an integral membrane protein present in urothelial plaques, and the murine UPIIIA knockout is associated with urothelial anomalies and vesicoureteral reflux. De novo UPIIIA mutations recently were identified in 4 of 17 patients with severe bilateral renal adysplasia. Methods: To evaluate the overall role of UPIIIA in human renal hypodysplasia pathogenesis, we performed UPIIIA mutation analysis in a cohort of 170 pediatric patients affected by severe unilateral or bilateral renal hypodysplasia. Eighty-one patients were affected by bilateral nonobstructive renal hypodysplasia; of these, 51 were without vesicoureteral reflux. Eighty-four patients presented with unilateral nonobstructive renal hypodysplasia, including 24 patients with unilateral multicystic dysplastic kidneys. Family history was positive in 11%. Results: Mutation analysis showed 2 heterozygous mutations not observed in 200 race-matched control chromosomes. In only 1 family was distribution of the UPIIIA mutation consistent with a disease-causing effect. This de novo missense mutation (Gly202Asp) was identified in a patient with unilateral multicystic dysplastic kidneys. The second (intronically located) mutation appeared unlikely to be disease causing because it did not segregate with an obvious disease phenotype in the affected family. Conclusion: Our results indicate that de novo mutations in UPIIIA can be involved in defective early kidney development, but probably constitute only a rare cause of human renal hypodysplasia in a minor subset of individuals. Am J Kidney Dis 47:1004-1012.

INDEX WORDS: Kidney hypoplasia; dysplasia; uroplakin IIIa; UPIIIA; mutation analysis; de novo mutation; vesicoureteral reflux.

Renal hypodysplasia is a common congenital defect of kidney development characterized by a decrease in nephron number, small overall kidney size, and maldeveloped renal tissue with lack of normal nephrogenic zones, primitive tubules, and metaphasic cartilage. One in 200 neonates presents with anomalies of the kidneys and/or urinary tract on renal ultrasound, and renal hypodysplasia is one of the prominent anomalies observed. Renal hypodysplasia is a major cause of pediatric renal insufficiency, accounting for more than 20% of children with end-stage renal disease, but little is known about its molecular pathogenesis. Because of the fortunate advances in renal replacement therapies, the majority of children with end-stage renal disease reach adulthood age today. Therefore, also adult nephrologists increasingly will be confronted with patients affected by renal hypodysplasia. Several lines of evidence suggest that genetic factors are involved in this disease, in particular, the observation that in approximately 10% of patients with renal hypodysplasia, careful evaluation of the family history shows familial clustering of malformations of the kidney and urinary tract. Moreover, numerous transgenic animal models for renal developmental genes present with a phenotype highly reminiscent of human renal hypodysplasia. To date, a number of human gene mutations were associated with hereditary renal hypodysplasia, including PAX2 (renal-coloboma syndrome), EYA1...
(branchio-oto-renal syndrome), and HNF-1β (renal cysts and diabetes syndrome), but mutations were identified in only a minority of patients.

Very recently, Jenkins et al identified heterozygous de novo mutations in the uroplakin IIIa (UPIIIa) gene (UPIIIA) in 24% of patients with nonobstructive bilateral renal hypodysplasia. This is the first report of human mutations affecting patients with renal hypodysplasia with such high frequency; 1 UPIIIA missense mutation and 2 mutations locating to the 3’ untranslated region (UTR) were identified in 4 of 17 patients with bilateral adysplasia.5

Uroplakins are a group of 4 integral membrane proteins (UPIa, UPIb, UPII, and UPIIIa, forming UPIa/UPII and UPIb/UPIII dimers) that are expressed by mammalian urothelia as their major differentiation product.6-8 Ablation of UPIIIa in the mouse model is associated with major changes in urothelial morphological characteristics and function, including absence of the typical superficial umbrella cell layer, decrease in apical urothelial plaque size, and compromised urothelial function as permeability barrier.9,10 Moreover, UPIIIa-deficient animals present with markedly enlarged ureterovesical orificia and vesicoureteral reflux (VUR); however, no human UPIIIa mutations have been identified in patients with isolated VUR to date.11-14

On the basis of the results of Jenkins et al5 and to evaluate the overall role of UPIIIA for human renal hypodysplasia pathogenesis, we performed UPIIIA mutation analysis in a cohort of 170 patients with renal hypodysplasia. Eighty-one of these patients presented with severe bilateral nonobstructive renal hypodysplasia similar to patients included in the initial report on human UPIIIA gene mutations.

METHODS

Patients

One hundred seventy children with renal hypodysplasia phenotype were enrolled for genetic mutation analysis in a European multicenter study including renal hypodysplasia patients of the Effect of Strict Blood Pressure Control and ACE Inhibition on the Progression of CRF in PEdiatric Patients (ESCAPE) trial. Forty percent of study patients were of Western origin; 30%, Eastern European origin; and 30%, Turkish origin.

Renal hypodysplasia is defined by the presence of small kidneys (<3rd percentile) and/or maldeveloped renal tissue on renal ultrasound. Sonographic criteria for maldeveloped renal tissue include lack of corticomedullary differentiation and optional proof of renal cysts.15 When required, renal ultrasound was followed by cystourethrography under mic- turition to detect posterior urethral valves in boys and VUR in both sexes. If necessary, scintigraphic analyses were conducted to analyze left and right kidney function separately. Rarely, magnetic resonance urography was performed to precise the anatomic malformations. Patients with ureteral anomalies and/or isolated VUR, but kidneys of normal sonographic size and structure, were excluded from the study. Sixty patients with primary bladder (n = 11) or urethral abnormalities (n = 59) including posterior urethral valves were screened separately for UPIIIA gene mutations. Clinical characteristics of the study cohort are listed in Table 1.

DNA from the parents was analyzed if positive mutation results were obtained in a patient. Clinical information from family members was collected when possible. The study was approved by the ethical committees of all participating institutions.

Table 1. Clinical Characteristics of the Patient Cohort

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>11.0 ± 6.3 (n = 157)</td>
</tr>
<tr>
<td>Sex (men/women; %)</td>
<td>58/42 (n = 163)</td>
</tr>
<tr>
<td>NRF/CRF/ESRD (continuous dialysis, NTPL) (%)</td>
<td>15/54/31* (n = 106)</td>
</tr>
<tr>
<td>Patients with positive family history for renal disease (%)</td>
<td>11 (n = 113)</td>
</tr>
<tr>
<td>Patients with unilateral nonobstructive RHD (%)</td>
<td>84</td>
</tr>
<tr>
<td>Unilateral nonobstructive RHD without VUR (%)</td>
<td>66</td>
</tr>
<tr>
<td>Unilateral agenesis (%)</td>
<td>24</td>
</tr>
<tr>
<td>Unilateral MCDK (%)</td>
<td>24</td>
</tr>
<tr>
<td>Isolated unilateral nonobstructive RHD with VUR</td>
<td>18</td>
</tr>
<tr>
<td>Patients with bilateral nonobstructive RHD (%)</td>
<td>81</td>
</tr>
<tr>
<td>Bilateral nonobstructive RHD without VUR (%)</td>
<td>61</td>
</tr>
<tr>
<td>Bilateral nonobstructive RHD with VUR (%)</td>
<td>20</td>
</tr>
<tr>
<td>Patients with unilateral obstructive RHD (%)</td>
<td>4</td>
</tr>
<tr>
<td>Patients with bilateral obstructive RHD (%)</td>
<td>1</td>
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</table>

Abbreviations: NRF, normal renal function; CRF, chronic renal failure; ESRD, end-stage renal disease; NTPL, kidney transplantation; RHD, renal hypodysplasia.

*Twenty-nine percent of all patients with ESRD were on continuous dialysis therapy and 71% received a renal transplant.
centers, and informed assent and/or consent for genetic screening was obtained from patients and/or parents, as appropriate.

One hundred race-matched individuals, unrelated to the patients, served as controls.

**Mutation Screening**

Genomic DNA was extracted from peripheral-blood leukocytes by using standard methods. Overlapping sets of primers were used to amplify by means of polymerase chain reaction (PCR) the coding sequences of **UPIIIA**, including the flanking splice sites. Primer design was accomplished by applying the software primer3_www.cgi, version 0.2 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi); primer sequences are available upon request. The 3’ UTR of **UPIIIA** was PCR amplified as described by Jenkins et al. to provide comparable results. Mutation screening was performed by using single-strand conformation polymorphism (SSCP) analysis (Multiphor II; Pharmacia Biotech, Uppsala, Sweden) and completed by direct sequencing on both strands of PCR-amplified mutation-carrying fragment of the affected patients. Normal band patterns in controls confirmed the PCR products were compared by means of SSCP to the PCR-amplified mutation-carrying fragment of the affected patient. Normal band patterns in controls confirmed the mutation prevalence only in the patient.16

Gene proof of paternity in family 1 was accomplished by using PCR amplification and analysis of informative microsatellite markers (D7S486, D14S280, D16S420, IVS17BTA [CFTR], KG8 [PKD1], D15S128, D15S122, D18S161, and D14S1007) in all family members.

**Statistical Analyses**

Statistical analyses of protein structures and amino-acid composition, sequence alignments, and similarity searches were conducted by using software and databases provided by Infobiogen (www.infobiogen.fr) and the National Center for Biotechnical Information (www.ncbi.nlm.nih.gov). Bioinformatic statistical analysis of intronic sequence variations was performed by using Automated Splice Site Analysis (https://splice.cmh.edu).

**RESULTS**

**UPIIIA** mutation analysis in 170 patients with renal hypoplasia showed 2 heterozygous **UPIIIA** mutations not identified in 200 race-matched control chromosomes: 1 missense mutation resulting in an amino-acid exchange (Gly202Asp) and a second mutation located in an exon-near intronic region (g→a (489-12)). Both affected patients were of Turkish origin; none presented with extrarenal symptoms. In only family 1, distribution of the **UPIIIA** mutation was suggestive of a disease-causing effect, as described next (Fig 1).

In the index patient (no. 1) of family 1, affected by a unilateral multicystic dysplastic kidney (MCDK; Fig 2), a G→A nucleotide exchange was identified at position 605. This nucleotide exchange represents a typical mutational event in a GpC-rich sequence stretch and results in an amino-acid exchange from glycine to asparagine at position 202 (Gly202Asp; Fig 3), a residue of the extracellular domain highly conserved in different species throughout evolution (Fig 4). This exchange replaces the small amino-acid glycine by a large and negatively charged amino acid that is likely to affect the secondary protein structure. In the affected patient, MCDK was diagnosed on only the right side, whereas the left kidney showed normal size and structure in renal ultrasound. The patient was born in 2001, and at the age of 4 years, serum creatinine level was 0.28 mg/dL (25 μmol/L) and blood urea nitrogen level was 14.9 mg/dL (5.3 mmol/L), indicating normal renal function. Serum electrolyte levels were normal, and urinalysis results were unremarkable. Blood pressure was in the normal range (mean, 120/80 mm Hg). No additional diseases or extrarenal malformations were detected. Interestingly, family analysis for the Gly202Asp missense mutation showed that no other family member was carrying this mutation, indicating its de novo appearance, as seen in patients described by Jenkins et al.5 Paternity was proven as described. As can be expected, no other family member was affected by renal malformations (2 sisters, 1 brother, and the parents).

The second mutation, a heterozygous G→A conversion, is intronically located (12 nucleotides, 5’ to exon 4). The affected index patient (no. 2) also presented with unilateral MCDK. Both brother and sister shared this heterozygous sequence variant, but renal ultrasound results were normal in these siblings and all other family members. Mutational analysis was negative in the mother, and, unfortunately, no DNA sample was available for genetic analysis of the father because he had died in an accident. Most probably, the father transmitted the mutation to all siblings presenting with the heterozygous G→A conversion. Bioinformatic statistical analysis showed that normal **UPIIIA** RNA splicing mechanisms will not be disturbed by this sequence variation. We cannot completely exclude that minor kidney anomalies were missed by ultra-
sound in the heterozygous siblings of the index patient. However, because of the intronic nature of this sequence variation and lack of segregation with an obvious disease phenotype in the affected family, we doubt its nature as a disease-causing mutation.

In summary, 2 heterozygous sequence variants were identified in a cohort of 170 patients with renal hypodysplasia, but only 1 mutation was suggestive of linkage to the disease phenotype, constituting a de novo mutation, as described by Jenkins et al for *UPIIIA* mutations in patients with severe renal hypodysplasia. Following these observations, the mutation detection rate amounts to 0.6% in the present study. One heterozygous de novo *UPIIIA* gene mutation (Gly202Asp) was identified in the index patient (no. 1) with unilateral MCDK. In this family, history for renal disease was negative, and the mutation was absent in the parents and siblings of the patient. Nineteen patients with unilateral MCDK also were studied by Jenkins et al in the initial study, but no *UPIIIA* mutations were identified in these individuals. As suggested by the same investigators, the observation of a de novo mutation strongly supports its implication in the renal malformation observed in this family.

DISCUSSION

To corroborate the recently suggested major role of *UPIIIA* mutations for renal adysplasia, we screened 170 patients with renal hypodysplasia, including 81 patients with severe bilateral renal hypodysplasia, for mutations in this gene. In contrast to results of the study by Jenkins et al showing de novo *UPIIIA* mutations in 24% of patients with bilateral renal hypodysplasia, the mutation detection rate was low in our study cohort. One heterozygous de novo *UPIIIA* gene mutation (Gly202Asp) was identified in the index patient (no. 1) with unilateral MCDK. In this family, history for renal disease was negative, and the mutation was absent in the parents and siblings of the patient. Nineteen patients with unilateral MCDK also were studied by Jenkins et al in the initial study, but no *UPIIIA* mutations were identified in these individuals. As suggested by the same investigators, the observation of a de novo mutation strongly supports its implication in the renal malformation observed.
in the affected patient. The impact of de novo mutations for rare hereditary diseases formerly was analyzed, and it generally is agreed that de novo mutations are highly suggestive of being causative. Still, the association with the disease-phenotype might be incorrect. Taking all data together, however, the observation of de novo \textit{UPIIIA} mutations in 5 different patients of diverse ethnic origins affected by renal hypodysplasia seems unlikely to be accidental.

The intronic sequence variation (g→a [489-12]) identified in the present study also was detected in healthy family members of the index patient (no. 2) and does not affect normal \textit{UPIIIA} splicing mechanisms in in silico analysis. As discussed, it probably constitutes a rare polymorphic variant with an allele frequency of less than 0.5%. No other studied \textit{UPIIIA} gene polymorphism showed significant differences between the patient and control cohorts, and these results are similar to results of Jiang et al. In the respective study, only the Pro154Ala polymorphism had been related with low significance to primary nonsyndromic VUR. However, patients with isolated primary VUR and kidneys of normal size and structure were excluded from our study cohort (as described in Methods). Larger sample sizes are necessary to deliver results of clinical importance in a reliable polymorphism-association study.

The unusual description of de novo mutations in the 3' UTR of the \textit{UPIIIA} gene in the initial report of Jenkins et al promoted analysis of the identical genomic segment in our patient cohort, but no 3' UTR mutations were identified in the 170 patients of the present study. Given sensitivity of the SSCP screening method of 80% to 90%, a very low number of sequence variations might have been missed. To date, mutations affecting regulatory sites within the 5' and 3' UTRs were described for few human disorders, eg, arrhythmogenic right ventricular cardiomyopathy and ocular cone dystrophy.
These nucleotide exchanges are predicted to alter transcript levels, and a similar mechanism might hold true for UPPIIA 3' UTR mutations. The greater mutation detection rate in the cohort of the initial study probably is caused by biased patient selection.

The 2 patients with de novo UPPIIA Pro273Leu mutation in the initial study of Jenkins et al\textsuperscript{5} presented with severe VUR and associated hydro nephrosis in addition to renal hypodysplasia. Interestingly, no UPPIIA mutations were observed in a large cohort of 76 patients with VUR, among these, 59 patients of families with more than one affected sibling,\textsuperscript{13} or in a second study including 25 patients with primary VUR and a positive family history for VUR.\textsuperscript{12} Both studies included the 3' UTR fragment analyzed by Jenkins et al.\textsuperscript{5} It can be expected that an important percentage of these patients with VUR also presented with kidney hypoplasia/dysplasia, although probably with varying degrees of severity. Genetic data collected to date allow the conclusion that (de novo) UPPIIA mutations seem to be a rare event, particularly affecting children with severe congenital renal hypodysplasia, including MCDK.

Some investigators suggested that lack or dysfunction of uroplakins are associated with functional obstruction of urine flow,\textsuperscript{20} and, interestingly, the cause of MCDK is related to ureteral atresia.\textsuperscript{21,22} However, the relation of bilateral renal adysplasia (observed in the initial study of patients affected by UPPIIA mutations) to MCDK remains unclear. Renal adysplasia falls into the category of kidney agenesis, aplasia, and dysplasia with or without cysts. Patients included in the initial report of Jenkins et al\textsuperscript{5} presented with typical malformations of the congenital anomalies of the kidney and urinary tract (CAKUT) complex, and 1 patient with additional severe genitourinary malformations. Following the CAKUT theory, early defects in ureteral branching and mesenchyme induction result in malformation/dysplasia of the kidneys and urinary tract. Results of the present study suggest that MCDK also is related to the CAKUT complex because mutations in the same gene (UPPIIA) apparently can be associated with both phenotypes. A similar observation recently was reported in a family with positive PAX2 mutation results in individuals affected by MCDK.\textsuperscript{23} PAX2 mutations are associated with renal-coloboma syndrome, characterized by ocular coloboma and kidney hypoplasia or dysplasia. For the first time, PAX2 mutations also were identified in MCDK, and a role for early ureteric obstruction and subsequent
renal maldevelopment is discussed in this work. In summary, different mutation analysis studies point to a close relation of MCDK to the CAKUT malformation complex, including renal hypodysplasia, and its cause possibly is explained as a resulting phenotype after CAKUT-associated ureteral obstruction.

To date, it is unknown why heterozygous UPIIIA alterations are associated with severe disruption of human nephrogenesis. Immunohistochemical studies provided evidence of early antenatal UPIIIA expression in the luminal epithelial layer of the urogenital sinus at 7 weeks and in the bladder, ureter, and renal pelvis at 13 weeks of gestation, suggesting functional relevance for early organogenesis. Murine UPIIIa and UPII knockout models are both affected by a severe renal phenotype, including VUR, hydronephrosis, and renal dysfunction. It was shown that uroplakins form heterodimers in urothelial plaques, in particular, UPII with UPIa and UPIIIa with UPIb. Although UPIa and UPIb belong to the tetraspanin protein family, characterized by 4 membrane spanning domains, both UPII and UPIIIa share a single transmembrane domain and extracellular N-termini (Fig 3). Tetraspanins form large protein networks that regulate cell motility, aggregation, and signaling. It is tempting to speculate that uroplakins participate in the formation of transmembrane networks during early organogenesis, with an important role for cell-cell contacts during normal kidney development. A role of UPIIIa for intracellular signal transduction is supported by the recent identification of xUPIII in Xenopus laevis, a lipid/membrane raft-associated protein that is tyrosine-phosphorylated upon egg fertilization and highly homologous to mammalian UPIIIa. Interestingly, xUPIII also is expressed strongly in the Xenopus urinary tract and kidneys. After fertilization, xUPIII becomes rapidly phosphorylated on tyrosine residue 249, which locates in the carboxyl-terminal cytoplasmic tail of the molecule. Raft localization and tyrosine phosphorylation of xUPIII can be reconstituted in a cell model by coexpression of xUPIII and a Xenopus tyrosine kinase, and this interaction can be blocked by an antibody against the extracellular domain of xUPIII. Interestingly, the Pro273Leu missense mutation identified in the study of Jenkins et al locates to close proximity of the human UPIIIa tyrosine residue Tyr266 on the intracellular side of the protein. This phosphorylated Thy266 residue is equivalent to the phosphorylated Xenopus residue Tyr249 (Fig 4), and it can be speculated that the pathogenicity of the Pro273Leu amino acid exchange is related to disturbed phosphorylation mechanisms that are followed by altered signal transduction.

The Gly202Asp missense mutation identified in the present study locates to a stretch of high homology between UPII and UPIIIa, and, interestingly, Gly202 is part of a 3-amino-acid “conserved domain” (Gly202-Arg203-Arg204) that was described first by Wu and Sun in 1993. Very recently, it was shown that the corresponding “conserved domain” in xUPIII (Gly186-Arg187-Arg188) seems to be a proteolytic site of the sperm-derived protease in the frog UPIII molecule. These investigators showed that activation of Xenopus eggs by a protease is accompanied by tyrosine phosphorylation of xUPIII and also partial digestion of xUPIII in vitro and in vivo. A synthetic xUPIII-Gly-Arg-Arg peptide (corresponding to Gly202-Arg203-Arg204 in human UPIIIa) inhibits proteolysis and tyrosine phosphorylation of xUPIII.

From these observations, it might be deduced that human UPIIIa also could be involved in intracellular signal transduction mechanisms, and transduction characteristics are altered by the Gly202Asp UPIIIA missense mutations because this mutation seems to affect a critical proteolytic protein site. Therefore, the human genetic findings of the present work offer further evidence that signal transduction mechanisms into urothelial cells of the human renal tract might have a role in early kidney development. Gly202Asp and Pro273Leu are the only UPIIIA mutations identified in humans to date that directly affect the primary protein structure, and in addition to in vitro studies, transgenic animal models expressing mutant UPIIIa proteins will provide a useful basis for additional functional analyses.

In summary, heterozygous de novo UPIIIA mutations were detected in 0.6% of patients with renal hypodysplasia. These data suggest that UPIIIA gene mutations can be involved in defects of early kidney development, but probably constitute a rare cause of human renal hypodysplasia in only a very small subset of affected
individuals. Genetic counseling for this small number of patients and their family members is demanding. In general, recurrence risk for de novo mutations is considered to be very low because they occur most often in the index patient only. The index patient will be transmitting the mutation to 50% of his offspring, but the penetrance of UPIIIA gene mutations remains unknown to date.

Because UPIIIA gene mutations do not represent the major cause of renal hypodysplasia, other genetic and/or nongenetic factors, including mutations in other renal developmental genes, will have to be analyzed in patients with UPIIIA-negative renal hypodysplasia.

ACKNOWLEDGMENT

The authors thank the patients and their families for participating in this study, Dr Bart Janssen (Institute of Human Genetics, University of Heidelberg, Germany) for genetic proofs of paternity, and Alexandra Ochs for technical support.

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APPENDIX

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