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Mutation analysis of the *Uromodulin* gene in 96 individuals with urinary tract anomalies (CAKUT)

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Abstract

Uromodulin (*UMOD*) mutations were described in patients with medullary cystic kidney disease (MCKD2), familial juvenile hyperuricemic nephropathy (FJHN), and glomerulocystic kidney disease (GCKD). *UMOD* transcription is activated by the transcription factor *HNF1B*. Mutations in *HNF1B* cause a phenotype similar to FJHN/GCKD but also congenital anomalies of the kidney and the urinary tract (CAKUT). Moreover, we recently detected *UMOD* mutations in 2 patients with CAKUT. As *HNF1B* and *UMOD* act in the same pathway and cause similar phenotypes we here examined, whether *UMOD* mutations would be found in patients with CAKUT.

Mutation analysis of *UMOD* was performed in 96 individuals with CAKUT by direct sequencing of exons 4 and 5 and by heteroduplex analysis following CEL I digestion assay of the exons 3 and 6–12.

The mean age of patients was 11.4 years and in 36.4% of patients the family history was positive for CAKUT. In the CEL I assay 12 aberrant bands were detected in 103 of 960 PCR products and were sequenced. Two previously known and eight new SNPs were detected. As no *UMOD* mutations were identified in these 96 patients with CAKUT, *UMOD* mutations do not seem to be a significant cause of CAKUT in this cohort.

Keywords

Uromodulin; Tamm-Horsfall protein; urinary tract malformation; CAKUT; mutation analysis

INTRODUCTION

The *Uromodulin* (*UMOD*) gene encodes Tamm-Horsfall protein (THP), which is the most abundant urinary protein in humans [1]. *UMOD* mutations were found in a large variety of

different kidney diseases: (i) medullary cystic kidney disease type 2 (MCKD2) [2], (ii) familial juvenile hyperuricemic nephropathy (FJHN) [3,4], and (iii) glomerulocystic kidney disease (GCKD) [4]. All of these disorders are inherited in an autosomal dominant mode and represent a progressive tubulo-interstitial nephropathy. Multiple groups including our own have confirmed mutations in *UMOD* as causing MCKD2, FJHN and GCKD [3–6].

We analyzed the *UMOD* gene for mutations in a cohort of 96 patients with congenital anomalies of the kidney and the urinary tract (CAKUT) for following reasons:

- i. *UMOD* is strongly expressed in the newborn mouse kidney and is regulated by the transcription factor *Brn1* during renal development [7]. Homozygous *Brn1* knock-out mice die postnatally due to renal insufficiency and renal hypoplasia.
- ii. Interestingly, we detected in our cohort of patients with *UMOD* mutations two individuals, who also had associated CAKUT. One individual had right renal hypoplasia and the other individual had vesiculo-ureteral reflux [8]. No other related urinary tract malformations in patients with *UMOD* mutations have been published so far.
- iii. Gresh et al showed that several genes responsible for cystic kidney disease (e.g. *PKD1*, *PKD2*, *PKHD1*, *NPHP1*), including *UMOD*, are under transcriptional control of *HNF1B* and form a transcriptional network [9]. A recent publication reports that 29% of all fetal bilateral hyperechogenic kidneys are caused by mutations in *HNF1B* [10]. Salomon et al found *HNF1B* mutations in three out of 105 individuals with sporadic renal hypoplasia/dysplasia [11]. Interestingly, mutations in the *HNF1B* gene can also result in a phenotype similar to MCKD2, including renal cysts and renal failure (RCAD syndrome) [12]. Besides renal cysts and maturity onset diabetes of the young type 5 (MODY5), patients with mutations in *HNF1B* have also various renal abnormalities, including renal agenesis, renal dysplasia [12], and hypoplastic GCKD [13]. Moreover, a phenotype similar to FJHN with severe hyperuricemia, which is usually caused by *UMOD* mutations, was published due to *HNF1B* mutations [14].

Taking the phenotypical similarities between patients with mutations in *UMOD* and *HNF1B* into account and recognizing that *Brn1* and *HNF1B* are transcription factors, which regulate *UMOD* and result in a CAKUT phenotype when mutated, we examined whether *UMOD* mutations are a frequent cause of CAKUT in a world-wide cohort of 96 CAKUT individuals. No mutations were detected. We conclude that *UMOD* mutations are not a frequent cause of CAKUT in this cohort.

METHODS

Patients

We ascertained 96 individuals with urinary tract malformations from 20 pediatric nephrology units in 5 European countries (Germany, Macedonia, United Kingdom, Hungary, Serbia) and the United States. The patients were included in the study, if a diagnosis compatible with congenital abnormality of the kidney or the genital tract was established by a pediatric nephrologist. Presenting symptoms encompassed urinary tract infection, prenatal ultrasound, work up for abdominal pain, abdominal masses, hypertension and chronic renal failure. Ultrasound, voiding cysturethrography (VCUG), intravenous pyelogram or scintigraphy was performed in order to establish the correct diagnosis. Further clinical characteristics of the 96 individuals are shown in Table 1. The family history was interpreted as positive, if one or more closely related individuals also were identified with a CAKUT phenotype. The glomerular filtration rate (GFR) was calculated by using the Schwartz formula. The study was approved by the institutional review board of the

University of Michigan Medical School. All participating family members provided informed consent.

Mutational analysis of the *UMOD* gene

We used standard methods to isolate genomic DNA from peripheral blood samples according to the manufacturer's instructions (Puregene™, Gentra Systems). Mutational analysis of *UMOD* exons 4 and 5 was performed by exon PCR and direct sequencing of the *UMOD* gene, as most mutations have been identified in these 2 exons so far [2–6,8]. Primer sequences were used as described by Wolf et al [5]. PCR products were purified, sequenced and analyzed as previously described [15].

CEL I endonuclease preparation

Exons 1 and 2 do not encode translated parts of *UMOD*, they contain only 5' untranslated regions and were therefore not included in the analysis. Exons 3 and 6–12 were examined by exon PCR, CEL I endonuclease digest and heteroduplex analysis with consecutive sequencing of products yielding aberrant bands. Preparation of crude extract containing 30 kDa single strand specific endonuclease CEL I was purified from celery as described [16,17].

Heteroduplex formation and CEL I treatment

The CEL I endonuclease enzyme recognizes single base mismatches present in heteroduplex DNA and cleaves both strands. Heteroduplex DNA was obtained by heat-denaturing 2 µl of an exon-PCR amplified patient DNA sample. DNA fragments were denatured at 95°C for 10 min followed by gradual cooling. The reaction mixture was incubated at 45°C for 5 min, put on ice, and stopped by mixing with glycerol-containing (30%) loading buffer supplemented with EDTA (final concentration 250 mM) followed by a 1.5% agarose gel electrophoretic separation of the digested fragments for 1 hour at 150V. Samples showing aberrant bands were purified and directly sequenced.

RESULTS

We analyzed the genomic DNA of 96 patients from 96 different families with CAKUT from Central Europe, Eastern Europe and the United States of America for *UMOD* sequence variations. Mean age at examination was 11.4 years (range 3 months to 44 years), the cohort consisted of 60% males and estimated mean GFR at examination was 89.6 ml/min/1.73 m² (range 13 to 167 ml/min/1.73 m²) (Table 1). In 36.4% of all patients a positive family history was found. The clinical data on identified CAKUT phenotypes is shown in Table 1.

Exons 3 and 6 to 12 were analyzed by CEL I heteroduplex analysis. Exons 4 and 5, in which 95% of all *UMOD* mutations were found so far, were analyzed by direct sequencing, in order to reduce the possibility of missing a mutation by CEL I heteroduplex analysis. The CEL I assay resulted in 12 different aberrant band patterns in 103 samples out of a total of 960 PCR products. These 103 samples were subsequently sequenced (Table 2).

Out of the 12 different aberrant band patterns of the CEL I heteroduplex analysis two aberrant band patterns represented two known single nucleotide polymorphisms (SNPs): the SNPs rs36060036 and rs4506906 [18] were identified in exons 3 and 6, respectively. Only for SNP rs4506906 in exon 6 an allele frequency was given. Published heterozygosity for rs4506906 is 0.516 in a European population. We identified a heterozygosity of 0.447 in our cohort (Table 2).

Out of the 12 different aberrant band patterns of the CEL I heteroduplex analysis eight aberrant band patterns represented novel single nucleotide polymorphisms (SNPs): in 23 patients new sequence variants were found in exons 3, 6, 7, 8, 9, and 12, respectively (Table 2). Out of the eight novel SNPs four did not affect the encoding region of *UMOD*: three of them were located in the 5'UTR (2 in exon 3) and one was located in the 3'UTR (1 in exon 12) and did not have an effect on the encoded amino acid sequence (see Table 2).

Out of the eight novel SNPs four SNPs are located in the encoding region of *UMOD*. Two of these SNPs in exons 7 and 8 were found to result in the same amino acid (P428P and Y486Y, respectively). Two of the SNPs located in the encoding region resulted in an altered amino acid. SNP c.1372G>T (V458L) in exon 8 resulted in the amino acid Leucine instead of Valine. We found this variant in 4 out of 96 patients in this study and in 4 out 93 healthy controls in a previous study. Thus, we concluded, that this sequence variant represents a SNP. The second novel SNP resulting in an altered amino acid was c.1124G>A in exon 6, which resulted in the amino acid Glutamine instead of Arginine (R375Q). Both parents of this patient had CAKUT and thus segregation studies were not informative. However, the evolutionary conservation of Glutamine (Q) at this position was found to be poor as there is a Glutamine residue at this position in rabbit, armadillo and opossum. Because of the poor evolutionary conservation we concluded that this sequence variant represents another rare SNP. Two out of the 12 aberrant band patterns in exons 7 and 9 did not show any sequence variation and were interpreted as an artifact.

The mutation analysis of exons 4 and 5 by direct sequencing did not detect any mutations, but included the analysis of 3 known SNPs (2 in exon 4 and 1 in exon 5). For two of them no population frequency was given. For SNP rs13335818 in exon 4 the population frequency matched very well the expected numbers given by the NCBI SNP database (Table 2). In conclusion no mutation, which is accountable for the CAKUT phenotype, was detected.

DISCUSSION

Developmental abnormalities of the kidney are diverse, including renal agenesis, multiple ureters, renal hypoplasia and dysplasia, each of which corresponds to defects at a particular stage of development [19]. Multiple genes including *ROBO2*, *EYA1*, *SIX1*, *SIX2*, *SIX5*, *BMP4* and *PAX2* have been identified to be responsible for CAKUT [20–25]. In addition, identical mutations in the same gene were found to result in diverse phenotypes due to the stochastic nature of developmental defects [26]. Mutations in *HNF1B* can either cause a FJHN/GCKD like phenotype, as caused by mutations in *UMOD* or can result in CAKUT [10,11]. As we had previously detected *UMOD* mutations in two patients with MCKD2 who also had CAKUT, we here examined 96 patients with CAKUT for mutations in *UMOD* [8]. In the 96 patients we did not find any *UMOD* mutations, which we think are causative for CAKUT. There may be different reasons why we did not find any causative mutations:

- i. Mutations in other genes causing CAKUT appear to be very rare. Weber et al. performed mutational analysis in 466 CAKUT patients and her group did not find mutations in over 80% of their patients in the *SALL1*, *PAX2*, *EYA1*, *SIX1*, and *HNF1B* genes and concluded, that multiple other genes may be involved in causing CAKUT [21].
- ii. In a recent study the sensitivity of the CEL I assay was published at 92% in detecting 73 out of 79 known nephrocystin (NPHP) mutations [17]. Even though sensitivity for the CEL I heteroduplex analysis appears to be high, we still may have missed sequence variations in the *UMOD* gene in theory. We attempted to reduce this risk by sequencing the exons 4 and 5 where 95% of all mutations are

located. Moreover, the detection of eight novel sequence variations in the remaining exons indicates a rather sensitive analysis by CEL I heteroduplex assay.

- iii. The *UMOD* patients with CAKUT had either vesico-ureteral reflux (VUR) or unilateral hypoplastic kidneys [8]. We here assembled a CAKUT patient cohort similar to the one used by Weber et al [21], taking the wide variety of CAKUT and the stochastic nature of developmental defects into account. Several studies had shown that identical mutations can result in wide range of CAKUT phenotypes. However, perhaps we should have examined only patients with VUR and hypoplastic kidneys, as these were the CAKUT symptoms of the two *UMOD* patients, in order to obtain a higher yield of mutations.

In this study we looked only for dominant sequence variations, as *UMOD* mutations are inherited in an autosomal dominant way. In order to obtain heteroduplexes that may be cut by CEL I, an equal concentration of a wild-type and an altered allele are combined and form a heteroduplex after denaturation and gradual cooling. As we were looking for heterozygous variations we analyzed only patient DNA and did not mix the DNA sample with another heterozygous DNA sample, which would have been necessary to detect homozygous SNP alterations. This experimental limitation does not allow us to give the frequency for homozygous SNPs (Table 2).

Altogether, we have examined 96 patients with CAKUT for sequence alteration in the *UMOD* gene and could not identify a mutation responsible for the CAKUT phenotype. We can not rule out the possibility that these genes would represent less than 1% of observed cases with CAKUT. It is also possible that there could be mutations in known or unknown regulatory elements of *UMOD*.

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Table 1

Synopsis of the clinical features of 96 individuals with CAKUT, analyzed for mutations in the *UMOD* gene.

Clinical Features	Value
Mean age at examination (yr)	11.4 year (range 3 months to 44 years)
Male/Female ratio	60/40%
Estimated GFR (ml/min/1.73m ²)	89.6 (range 13 to 167 ml/min/1.73 m ²)
Positive family history for renal disease	36.4%
Unilateral renal agenesis	15
Renal hypoplasia	16 unilat., 12 bilat.
Noncystic renal dysplasia	9 unilat., 6 bilat.
Cystic renal dysplasia	11 unilat., 11 bilat.
MCDK	15
Associated vesicoureteric reflux	
unilateral	23
Bilateral	19
I°	4
II°	10
III°	15
IV°	11
V°	3
Associated distal ureteric obstruction	7
Associated pelvoureteric obstruction	5
Associated duplicated collecting system	6
Associated ectopic kidney	5

Table 2

Summary of the detected shifts by the CEL assay and their corresponding sequencing findings. Previously published SNPs are given with their SNP database number¹.

Exon ²	Number of patients with detected identical shifts	Sequencing findings corresponding to shift (Amino acid change)	Allele Frequency	SNP database Frequency ¹
3	In 37 patients shifts with 3 different patterns were found:			
	3 patients	-47T>C, het (none)	C/T 3/96, T/T or C/C in 93/96	New sequence variant, no published data
	2 patients	-16delG, het (none)	-16delG/WT in 2/96, homozygous for -16delG or WT 94/96	New sequence variant, no published data
	32 patients	rs36060036 ¹ (none)	A/G 32/96, A/A or G/G in 64/96	No data available
4	Direct sequencing, no CEL assay performed	rs7193058 ¹ (C174C)	T/T 46, C/T 41, C/C 9	No data available
		rs13335818 ¹ (V264V)	C/C 60, C/T 34, T/T 2	C/C63, C/T 35, T/T 2
5	Direct sequencing, no CEL assay performed	rs28544423 ¹ (G295G)	G/G 62, A/G 31, A/A 3	No data available
6	In 44 patients shifts with 2 different patterns were found:			
	43 patients	rs4506906 ¹ (none)	C/T 43/96, T/T or C/C in 53/96	C/T 52, C/C 13, T/T 35
	1 patient	c.1124G>A, het (R375Q) ³	G/A 1/96, G/G or A/A in 95/96	New sequence variant, no published data
7	In 2 patients shifts with 2 different patterns were found:			
	1 patient	Normal		
	1 patient	c.1284C>G, het (P428P)	C/G 1/96, C/C or G/G in 95/96	New sequence variant, no published data
8	In 5 patients shifts 2 different patterns were found:			
	4 patients	c.1372G>T, het (V458L)	G/T 4/96, G/G or T/T in 92/96	Own studies showed G/T in 4/93 healthy controls
	1 patient	c.1458C>T, het (Y486Y)	C/T 1/96, C/C or T/T in 95/96	New sequence variant, no published data
9	In 4 patients shifts with 2 different patterns were found:			
	3 patients	Normal		
	1 patient	IVS7-43C>G, het ⁴ (none)	1/96, C/C or G/G in 95/96	New sequence variant, no published data
10	No shifts			
11	No shifts			
12	In 11 patients shifts with 1 pattern were found:			
	11 patients	c.2026C>T 3'UTR (none)	C/T 11/96, C/C or T/T in 85/96	New sequence variant, no published data

¹ <http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp>

² Exons 1 and 2 were not analyzed as they contain only 5'untranslated region.

³ Glutamine at position 375 is conserved in rabbit, armadillo and opossum.

⁴ Analysis by Genscan did not reveal a changed splicing. 3'UTR, 3' untranslated region