Biallelic Expression of Mucin-1 in Autosomal Dominant Tubulointerstitial Kidney Disease: Implications for Nongenetic Disease Recognition

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ABSTRACT

Background Providing the correct diagnosis for patients with tubulointerstitial kidney disease and secondary degenerative disorders, such as hypertension, remains a challenge. The autosomal dominant tubulointerstitial kidney disease (ADTKD) subtype caused by MUC1 mutations (ADTKD-MUC1) is particularly difficult to diagnose, because the mutational hotspot is a complex repeat domain, inaccessible with routine sequencing techniques. Here, we further evaluated SNAPSHOT minisequencing as a technique for diagnosing ADTKD-MUC1 and assessed immunodetection of the disease-associated mucin 1 frameshift protein (MUC1-fs) as a nongenetic technique.

Methods We re-evaluated detection of MUC1 mutations by targeted repeat enrichment and SNAPSHOT minisequencing by haplotype reconstruction via microsatellite analysis in three independent ADTKD-MUC1 families. Additionally, we generated rabbit polyclonal antibodies against MUC1-fs and evaluated immunodetection of wild-type and mutated allele products in human kidney biopsy specimens.

Results The detection of MUC1 mutations by SNAPSHOT minisequencing was robust. Immunostaining with our MUC1-fs antibodies and an MUC1 antibody showed that both proteins are readily detectable in human ADTKD-MUC1 kidneys, with mucin 1 localized to the apical membrane and MUC1-fs abundantly distributed throughout the cytoplasm. Notably, immunohistochemical analysis of MUC1-fs expression in clinical kidney samples facilitated reliable prediction of the disease status of individual patients.

Conclusions Diagnosing ADTKD-MUC1 by molecular genetics is possible, but it is technically demanding and labor intensive. However, immunohistochemistry on kidney biopsy specimens is feasible for nongenetic diagnosis of ADTKD-MUC1 and therefore, a valid method to select families for further diagnostics. Our data are compatible with the hypothesis that specific molecular effects of MUC1-fs underlie the pathogenesis of this disease.


Hereditary kidney diseases are under-recognized, even if the patient’s pedigree clearly suggests a genetic cause of the disease. Systematic analyses have shown that >30% of patients with ESRD will have first- or second-degree relatives who have also suffered from ESRD,1,2 which is often not recognized in clinical practice. This misperception leads to imperfect clinical handling and consultation of patients and families, and it sometimes leads to unjustified therapeutic attempts.

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If the patient’s pedigree is not recognized, autosomal dominant tubulointerstitial kidney diseases (ADTKDs) are particularly difficult to diagnose. Usually, the patients present with isolated renal insufficiency and bland urinary findings.3 Hence, nephrologists often decide not to perform a renal biopsy. However, even if a renal biopsy is taken, the pathologic reports usually consist of descriptive and unspecific findings, such as interstitial fibrosis and tubular atrophy (IF/TA), varying degrees of glomerulosclerosis, vasculopathy, and interstitial inflammation with generally negative immunostaining. These findings could suggest a tubulointerstitial nature of disease, but they are often misinterpreted as hypertensive nephropathy.

A number of candidate genes have been identified for ADTKD: UMOD (Online Mendelian Inheritance in Man [OMIM] *191845, #603860),4 MUC1 (OMIM *158340, #174000),5 HNF1B (OMIM *189907, #137920),6 REN (OMIM *179820, #613092),7 and SEC61A1 (OMIM *609213, #617056).8 All listed genes can be analyzed by standard Sanger sequencing and/or massive parallel sequencing, which pathogenic mutations have been detected is within a standard Sanger sequencing and/or massive parallel sequencing, but generally shorter than the wild-type protein, because the frameshift causes a translational stop codon shortly after the VNTR.5 The MUC1-fs has been immunostained in single frameshift protein (MUC1-fs) in the tubular cytoplasm of ADTKD-MUC1 kidneys, where wild-type mucin 1 is also readily detectable. Molecular diagnosis of ADTKD-MUC1 is possible but restricted to a limited number of laboratories. Immunohistochemistry on kidney biopsies may be a feasible method for nongenetic selection of patients for further diagnostics.

Significance Statement

Clinical diagnosis of hereditary tubulointerstitial kidney disease has always been a challenge. The subform ADTKD-MUC1 largely escapes from molecular diagnostics, because its mutational hotspot is located in an inaccessible repeat domain. Here, the authors re-evaluate the detection of MUC1 mutations by SNaPshot minisequencing and establish immunohistochemistry for the resulting frameshift protein in human kidney samples in comparison with mucin 1 from the wild-type allele. MUC1 frameshift protein accumulates in the tubular cytoplasm of ADTKD-MUC1 kidneys, where wild-type mucin 1 is also detectable. Molecular diagnosis of ADTKD-MUC1 is possible but restricted to a limited number of laboratories. Immunohistochemistry on kidney biopsies may be a feasible method for nongenetic selection of patients for further diagnostics.

METHODS

More details are in Supplemental Material.

Cell Culture and Reagents

HeLa cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Reagents were purchased from PAN-Biotec (Aidenbach, Germany), PAA Laboratories (Coelbe, Germany), or Sigma-Aldrich (Taufkirchen, Germany).

Cloning of MUC1 cDNAs

The cDNA coding for the human MUC1 gene including 22 tandem repeats (MUC1/22TR; a gift from O. Finn, Pittsburgh, PA) served as a backbone to generate the mutated MUC1-fs plasmid via site-directed mutagenesis.

Generation of Polyclonal Antibodies

Four rabbit polyclonal antibodies were commercially generated by Pineda Antibody Service (Berlin, Germany). Animals were immunized with a 20-amino acid peptide corresponding...
to MUC1-fs (NH₂-CHLGPGHQAGPGLHRPPSPR-CONH₂) and affinity purified with the column-coupled peptide.

**Human Histologic Samples**

Kidney biopsies were collected retrospectively. The study was approved by the local ethics committee (protocol no. 4103 and 181_15 Bc). Patients included in this study signed a written informed consent form, which included genetic analysis and the review of historical renal biopsies (Supplemental Table 1).

**Immunohistochemistry and Immunofluorescence Staining**

Paraffin sections (2 μm) were dewaxed and cooked for antigen retrieval before staining. Immunodetection was performed by AEC+ (DAKO) or DAB solution (ImmPACT DAB Peroxidase Substrate Kit; Vector Laboratories, Burlingame) with standard protocols. Detailed antibody information is in Supplemental Table 2.

**Molecular Genetics**

Proband’s DNA was subjected to SNaPshot minisequencing after selective enrichment of the mutated MUC1 repeat as described. Haplotype reconstruction of selected families was performed by microsatellite analysis, for which the primers and all resulting genotypes are provided in Supplemental Tables 3 and 4.

**RESULTS**

Detailed knowledge of the expression pattern of physiologic mucin 1 in the kidney tubular apparatus is fundamental for the understanding of ADTKD-MUC1. Therefore, we characterized the segmental expression of mucin 1 in the kidney. Figure 1A and Supplemental Figures 1 and 2 show immunohistochemistry or immunofluorescence for mucin 1 in healthy human kidneys. As has been reported previously, we find broad tubular expression of mucin 1, which reaches from the thick ascending limb (TAL) of the Henle loop to the lower collecting duct, whereas no expression can be found in the proximal tubule. The most widely used mucin 1 antibody VU4H5, which detects an epitope within the VNTR domain, gives mostly cytoplasmic signals. However, an antibody raised against the C-terminal end of mucin 1 (ab80952) detects its target strictly at the apical membrane of the tubules. Both observations are true for healthy kidneys as well as kidneys from patients with ADTKD-MUC1. Because only the C-terminal antibody specifically detects the protein product of the wild-type allele (for explanation refer to Figure 3A) in ADTKD-MUC1 kidneys, this antibody clearly shows that the native protein is similarly expressed even in affected kidneys. Of note, mucin 1 expressing tubular segments in the TAL partially shows overlapping expression of the Tamm Horsfall protein (Figure 1B), which is the established origin of ADTKD-UMOD.

Since the establishment of MUC1 SNaPshot minisequencing at our university, we have identified 17 families with ADTKD-MUC1, of which six families are regional in the direct service area of our academic nephrology (Supplemental Table 1). To date, we only test patients with a positive family history for ESRD over at least two successive generations (clinically compatible with ADTKD). We have analyzed approximately 150 individuals by SNaPshot minisequencing, identifying 72 patients with ADTKD-MUC1. In all analyzed patients, clinical presentation and inheritance patterns matched the SNaPshot results. To further evaluate this, we performed microsatellite analysis and reconstructed haplotypes around the MUC1 gene locus for three selected ADTKD-MUC1 families (Figure 2). All indicated individuals had been analyzed by SNaPshot minisequencing, where the microsatellite results were completely consistent for positive
and negative calls as well as for the clinical presentation (accepting the limitation of clinical judgement of young individuals). Nevertheless, the analysis has its pitfalls, because it is technically demanding and very labor intensive, which is why only a restricted number of laboratories worldwide offer testing on a routine basis. It would, therefore, be beneficial to develop an easier, possibly nongenetic assay, which could be applied on a broader level. To achieve this, we generated polyclonal antibodies against MUC1-fs using a recombinant peptide coded by one repeat of the frameshifted VNTR sequence as immunogen.

Figure 3A schematically depicts the allele products in patients with ADTKD-MUC1 and the antibodies used in this study, showing the region of their detection. Importantly, the antibody directed against the native VNTR (VU4H5) will usually also detect the product from a mutated allele of any patient with ADTKD-MUC1; the duplication mutation usually takes place after a succession of “normal” repeats, which can be immunodetected (one published exemption16). Thus, for selective detection of mucin 1 from the wild-type allele, an antibody was used that detects an epitope beyond the premature STOP signal from the mutated sequence (ab80952; C-terminal). Prudent application of these antibodies in conjunction with the generated antibodies against MUC1-fs (pAbx-fs) allows for selective detection of the protein products of both alleles from patients with ADTKD-MUC1.

HeLa cells were transfected with empty vector pcDNA3 (Ø), MUC1 (WT), and MUC1-fs (FS) coding constructs, where whole-cell lysates were subjected to immunoblotting (Figure 3B). All four polyclonal sera detect a species at approximately 75 kD, which is concordant with the signal from sheep reticuloocyte lysates (in vitro transcribed and translated) programmed
Figure 3. Specific detection of overexpressed and endogenous MUC1-fs with pAb3-fs via immunoblot. (A) Scheme of the wild-type and mutated allele products as they occur in patients with ADTKD-MUC1 (dark blue boxes represent the wild-type VNTR motif, and red
A-50
A-47
A-23
A-21
A-20
A-19
A-18
A-15
A-14
A-12
A-10
A-9
A-8
A-7
A-6
A-5
A-4
A-3
A-2
A-1
A-0

Table 1. List of human kidney samples

<table>
<thead>
<tr>
<th>Family No. and Identification of Individual</th>
<th>Sample Type</th>
<th>Year of Sampling</th>
</tr>
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<tbody>
<tr>
<td>A-29 (1&lt;sup&gt;st&lt;/sup&gt;)</td>
<td>Biopsy</td>
<td>2011</td>
</tr>
<tr>
<td>A-DT KD-0032</td>
<td>Biopsy</td>
<td>2009</td>
</tr>
<tr>
<td>A-DT KD-0033</td>
<td>Biopsy</td>
<td>2011</td>
</tr>
<tr>
<td>A-DT KD-0034</td>
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<tr>
<td>A-DT KD-0035</td>
<td>Biopsy</td>
<td>2007</td>
</tr>
<tr>
<td>A-DT KD-0036</td>
<td>Biopsy</td>
<td>1999</td>
</tr>
<tr>
<td>A-30 (2&lt;sup&gt;nd&lt;/sup&gt;)</td>
<td>Biopsy</td>
<td>2006</td>
</tr>
<tr>
<td>A-DT KD-0042</td>
<td>Biopsy</td>
<td>2006</td>
</tr>
<tr>
<td>A-DT KD-0043</td>
<td>Biopsy</td>
<td>2006</td>
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<tr>
<td>A-DT KD-0044</td>
<td>Biopsy</td>
<td>2006</td>
</tr>
<tr>
<td>A-12</td>
<td>Biopsy</td>
<td>2014</td>
</tr>
<tr>
<td>A-DT KD-0015</td>
<td>Biopsy</td>
<td>2014</td>
</tr>
<tr>
<td>A-15</td>
<td>Biopsy</td>
<td>2014</td>
</tr>
<tr>
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<td>Nephrectomy</td>
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</tr>
<tr>
<td>A-DT KD-0075</td>
<td>Biopsy</td>
<td>2009</td>
</tr>
<tr>
<td>A-23</td>
<td>Nephrectomy</td>
<td>2008</td>
</tr>
<tr>
<td>A-DT KD-0026</td>
<td>Nephrectomy</td>
<td>2008</td>
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<tr>
<td>A-47</td>
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<tr>
<td>A-DT KD-0077</td>
<td>Biopsy</td>
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<tr>
<td>A-DT KD-0078</td>
<td>Biopsy</td>
<td>2008</td>
</tr>
<tr>
<td>A-50</td>
<td>Biopsy</td>
<td>2014</td>
</tr>
</tbody>
</table>

Kidney samples (either biopsy or nephrectomy) from 17 individuals from eight independent families were collected and analyzed. ADTKD, autosomal dominant tubulointerstitial kidney disease.

Indicates the nomenclature of renamed families as cited in the work by Ekiçi et al. 10

with MUC1-fs constructs. Unfortunately, immunocytochemistry in overexpression experiments in any cell line did not deliver specific results, because all negative controls produced a similar signal (data not shown). Therefore, we aimed to characterize the endogenous expression of MUC1-fs and analyzed protein lysates from human primary tubular cells (hUPTs) generated from the urine of three affected individuals from independent ADTKD-MUC1 families. Because patient’s MUC1-fs will harbor wild-type and frameshifted repeats, both pAb3-fs and the wild-type VU4H5 antibodies are able to detect the identical protein (Figure 3C). Knowledge of the individual VNTR length and the position of the mutated repeat by single-molecule real time sequencing enables us to suggest the specific species marked by an asterisk. To further verify the specificity of these signals, extracts of hUPTs from patients with ADTKD-MUC1 were blotted, and the lanes of the membrane were cut into two halves, of which one half was incubated with pAb3-fs and the other one half was incubated with VU4H5. For detection, the divided membranes were realigned, and the two antibodies produced signals of identical size (Figure 3D), corresponding to those detected in Figure 3C. siRNA for MUC1 clearly reduces the signal of MUC1-fs in the hUPTs of all patients analyzed (Figure 3E). Together, these data show that the anti-MUC1-fs sera can specifically detect endogenous MUC1-fs by immunoblotting.

We next turned to evaluation of our polyclonal sera on tissues. For this purpose, we collected 17 historic kidney biopsies or kidney tissues from eight different ADTKD-MUC1 families (Table 1). Staining with all four polyclonals on serial sections of a kidney biopsy from a patient with ADTKD-MUC1 produced a specific cytoplasmic signal in distinct tubuli (Supplemental Figures 3 and 4). These data together with the information from overexpression results (Figure 3B) favored the serum number 3 in terms of signal to noise. Thus, for the following procedures, we predominantly used pAb3-fs. In our
negative controls are crucial in this setting. Therefore, we used preimmune sera from the polyclonals as well as secondary-only staining experiments (Figure 4A). Figure 4C shows immunohistochemical staining with pAb3-fs after preadsorption by the MUC1 peptide used for immunizing the rabbits. A “control peptide” used for generating a different antibody (“hSPAG4”) was used in parallel. Furthermore, we stained ten healthy (living donor transplanted kidneys) and 12 fibrotic human kidney biopsies (Figure 4B). All of these scenarios produced a convincing negative result in comparison with the positive controls. Merely some of the fibrotic kidneys can produce a faint unspecific background staining pattern, also in tubuli.

Having established specific staining for MUC1-fs in tissues from affected patients, we next analyzed all available tissues. Figure 5 shows the results for MUC1-fs from kidney biopsies of patients from three different families in comparison with consecutive sections probed with the wild-type mucin 1 VNTR antibody. Stained tubular sections were identical for both antibodies, where all patients with ADTKD-MUC1 show a clear positive signal for both proteins. A control sample from a patient with ADTKD-UMOD is completely negative for MUC1-fs. Similar to wild-type mucin 1, MUC1-fs expression is spread through the tubules, beginning at the TAL and reaching down to the distal collecting duct (Supplemental Figure 3).

To compare expression and localization of mucin 1 from both alleles in ADTKD-MUC1 kidneys, we stained sequential sections of kidney biopsies with pAb3-fs and the C-terminal ab80952. Figure 6A shows that identical tubular sections are stained for both allele products. Whereas wild-type mucin 1 is strictly apical, MUC1-fs is distributed throughout the cytoplasm. This separation in localization is nicely seen in double-labeling experiments detected by immunofluorescence (Figure 6B).

Figure 4. Immunodetection of MUC1-fs in human kidneys. (A) Immunohistochemical staining of MUC1-fs in human kidneys compared with the serum of the corresponding rabbit before immunization (preimmune) and secondary antibody only (ADTKD-0034). (B) Immunohistochemical staining of mucin 1 and MUC1-fs in human kidney biopsies (VU4H5 and pAb3-fs, respectively) from living donors before transplantation (healthy kidney) or fibrotic kidneys, where no specific MUC1-fs expression could be detected. Biopsy from a patient with ADTKD-MUC1 (ADTKD-0042) serves as positive control for staining. (C) Immunohistochemical staining of serial sections of an ADTKD-MUC1 kidney biopsy (ADTKD-0038) for MUC1-fs with preadsorption of pAb3-fs with “no peptide,” nonspecific “control peptide” (hSPAG4 peptide used for generation of hSPAG4 antibody22), or frameshift peptide used for immunization against MUC1-fs (“MUC1-fs peptide”).
Finally, we aimed to test the efficiency of immunodetection with pAb3-fs in a nonbiased experiment. We screened a tissue array derived from random nephrectomy samples of 30 patients resected between 2005 and 2010. These samples were not tumor nephrectomies; hence, we presumed that a major proportion might have CKD. However, clinical records

![Figure 5](https://www.jasn.org/BASIC-RESEARCH/29/2298-2309,2018-Biallelic-MUC1-Expression-in-ADTKD/images/figure5.png)

**Figure 5.** Mucin 1 and MUC1-fs expression in patients with ADTKD-MUC1. Immunohistochemical detection of MUC1-fs and mucin 1 expression in affected members of three ADTKD-MUC1 families (A-29 [ADTKD-0032], A-30 [ADTKD-0043] and A-33 [ADTKD-0048]). Family A-32 (ADTKD-UMOD) serves as a negative control; it shows no MUC1-fs expression, but it does show mucin 1 expression. *The nomenclature of renamed families as cited in ref. 10.

![Figure 6](https://www.jasn.org/BASIC-RESEARCH/29/2298-2309,2018-Biallelic-MUC1-Expression-in-ADTKD/images/figure6.png)

**Figure 6.** Distinct biallelic expression of mucin 1 and MUC1-fs in patients with ADTKD-MUC1. (A) Immunohistochemical detection of mucin 1 (C-term) and MUC1-fs (pAb3-fs) in a patient with ADTKD-MUC1 (ADTKD-0075). Preimmune serum serves as a control. (B) Immunofluorescent detection of mucin 1 (C-term) and MUC1-fs in tubules of a patient with ADTKD-MUC1 (ADTKD-0034; green, MUC1-fs; red, wild-type mucin 1).
Figure 7. Identification of patients with ADTKD-MUC1 by unbiased tissue array screening. (A) Immunohistochemical identification of two individual patients with ADTKD-MUC1 (ADTKD-0024 and ADTKD-0026) on a randomly spotted kidney array, applying pAb3-fs for...
were not reviewed. All but two patient samples produced a completely clean signal with pAb3-fs (Figure 7A). Clinical records of the two patients who produced positive signals for MUC1-fs were then reviewed. Indeed, the cause of renal failure was unknown, and a positive family history was recorded in both patients (Figure 7B). The patients were contacted, and they consented to genetic analysis and our study, where ADTKD-MUC1 was subsequently confirmed in both patients.

**DISCUSSION**

Ever since the chromosomal locus of ADTKD-MUC1 was identified in 1998–202 (at the time mostly called medullary cystic kidney disease type 1), continuative scientific work always seemed to be extremely challenging. Thus, it took another 15 years and very sophisticated analyses to identify the disease-causing gene with **MUC1**.5 Since then, very few publications have been able to confirm and moderately extend the findings.10,16,17,24 To date, however, no studies have been able to shed light on the biologic behavior of the mutated protein or provide understanding of the pathogenesis of the disease. Therefore, it is an important step to establish the detection and understand the fate of the wild-type protein of mucin 1 as well as the mutated protein MUC1-fs in human kidneys. This should expedite diagnostics, epidemiology, and eventually, understanding of the pathogenesis of the disease.

For several technical issues, definitive molecular genetics for ADTKD-MUC1 will possibly remain a challenging issue in the near future. Therefore, the existence of a nongenetic test to select patients for molecular genetics would be a great advantage. The immunohistochemistry for MUC1-fs described herein is widely applicable and seems feasible for clinical services. Standard clinical samples that were paraffin embedded and stored for many years were successfully analyzed with a basic protocol for immunohistochemistry. Overall, the immunohistochemistry for MUC1-fs showed an unflawed sensitivity for detecting the disease and a very high specificity, where only very few severely fibrotic kidneys displayed a faint signal in renal tubuli. Thus, we believe that this assay stands up to the requirements of a clinical test. Importantly, other applications, like immunocytochemistry or immunohistochemistry, on skin samples did not deliver specific results (data not shown), where skin sebaceous glands show a high degree of staining for MUC1-fs in any (also nonaffected) sample. This may be due to some of the unspecific immunoreactivity seen in the immunoblotting (Figure 3).

ADTKD with its distinct subtypes is thought to be a very rare disease. We would challenge this view, and we hypothesize that most families to date have not yet been correctly diagnosed. For technical reasons, this will be particularly true for ADTKD-MUC1. Staining of kidney tissue arrays for MUC1-fs at a much larger scale than used here (Figure 7) could give some indication of the true prevalence of ADTKD-MUC1 in a population of patients with CKD. However, only a standardized analysis with molecular testing on a large cohort of patients will give the true figure. To date, we have performed molecular testing of 47 families with the clinical definition of ADTKD, where 60% of positively tested ADTKD families were identified with **MUC1** as the most frequent subtype; the second most frequent was **UMOD** (28%). Of note, further testing of families with no mutation found within the ADTKD spectrum showed mitochondrially inherited tubulointerstitial kidney disease (MITKD; OMIM *59007025*) or autosomal dominant FG5S in some patients (INF2; OMIM *610982; *613237; data not shown). Some sporadic cases that were completely clinically compatible with ADTKD have been shown to be adult-onset nephropathia. Other studies have identified families with autosomal dominant forms of Collagen 4 disease in similar cohorts.26 Therefore, exact clinical separation of families into such disease entities can be very challenging, and genetic testing should consider a spectrum of diseases, in particular when a positive family history for ESRD exists.

Our studies of human ADTKD-MUC1 kidneys have a number of implications that may help to improve our understanding of the disease. First, mucin 1 is readily detectable in ADTKD-MUC1 kidneys (Figures 1 and 6). This is completely in line with the results of a homozygous knockout of Muc1 in mice that showed no spontaneous phenotype.27 Second, spatial control of expression of both alleles of MUC1 in the kidney is intact and comparable in terms of tubular segments. This clearly implies that the disease arises somewhere between the TAL and the distal collecting duct or in the complete area. Interestingly, a distinct part of the TAL shows overlapping expression of both mucin 1 and MUC1-fs with uromodulin (Figure 1, Supplemental Figures 1 and 3). Obviously, regional expression of mutant UMOD in the TAL is sufficient to cause severe IF/TA and renal failure. Whether the same could be true for ADTKD-MUC1 would be a speculation at this point. Third, our data confirm the sparse information published to
date that MUC1-fs is strongly expressed and accumulates in the tubular cytoplasm. This circumstance is very similar to the situation in ADTKD-UMOD, where mutant protein accumulates in the endoplasmic reticulum and leads to an unfolded protein response in humans and recent mouse models. A clear distinction between the two diseases is becoming more evident. In UMOD-associated disease, many different pathogenic mutations have been described, whereas all MUC1 mutations found to date effectively lead to the same aberrant protein. The resulting intracellular expression of MUC1-fs may, therefore, lead to a more specific effect, which results in cellular harm. It would be of great scientific interest to identify these putative mechanisms. Because renal IF/TA is a common feature of progressive renal disease in general, identification of new molecular mechanisms leading to IF/TA may improve our understanding of CKD. Perhaps these studies will also help to develop novel avenues of therapeutic intervention.

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DISCLOSURES

None.

REFERENCES


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