Renal phenotypic investigations of megalin-deficient patients: novel insights into tubular proteinuria and albumin filtration*

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ABSTRACT

Background. The reabsorption of filtered plasma proteins, hormones and vitamins by the renal proximal tubules is vital for body homeostasis. Studies of megalin-deficient mice suggest that the large multi-ligand endocytic receptor megalin plays an essential role in this process. In humans, dysfunctional megalin causes the extremely rare Donnai-Barrow/Facio-Oculo-Acustico-Renal (DB/FOAR) syndrome characterized by a characteristic and multifaceted phenotype including low-molecular-weight proteinuria. In this study, we examined the role of megalin for tubular protein reabsorption in humans through analysis of proximal tubular function in megalin-deficient patients.

Methods. Direct sequencing of the megalin-encoding gene (LRP2) was performed in a family in which three children presented with classical DB/FOAR manifestations. Renal consequences of megalin deficiency were investigated through immunohistochemical analyses of renal biopsy material and immunoblotting of urine samples.

Results. In the patients, a characteristic urinary protein profile with increased urinary excretion of vitamin D-binding protein, retinol-binding protein and albumin was associated with absence of, or reduced, proximal tubular endocytic uptake as shown by renal immunohistochemistry. In the absence of tubular uptake, urinary albumin excretion was in the micro-albuminuric range suggesting that limited amounts of albumin are filtered in human glomeruli.

Conclusions. This study demonstrated that megalin plays an essential role for human proximal tubular protein reabsorption and suggests that only limited amounts of albumin is normally filtered in the human glomeruli. Finally, we propose that the characteristic urinary protein profile of DB/FOAR...
INTRODUCTION

Megalin is a 600 kDa multi-ligand endocytic receptor belonging to the LDL-receptor family (Supplementary data, Figure S1A) [1]. Megalin is encoded by the large LRP2 gene and mutations of LRP2 have been reported to cause the multifaceted Donnai-Barrow/Facio-Ocular-Acustico-Renal Syndrome (DB/FOAR syndrome) [OMIM # 222448] [2, 3]. DB/FOAR is an extremely rare, autosomal recessive inherited syndrome with less than 30 cases reported worldwide. DB/FOAR patients generally present with characteristic facial features such as hypertelorism, down-slanting palpebral fissures, tall broad forehead, low-set ears and functional deafness, including high-grade myopia, sensorineural hearing loss and low-molecular-weight proteinuria; however, great phenotypic variation has been reported [2, 4].

Consistent with the diverse and numerous clinical manifestations of these patients, megalin is expressed in several absorptive epithelia such as the visceral yolk sac, placenta, ciliary epithelium and the proximal tubules [5]. Our current knowledge of megalin physiology is mainly based on murine studies [6, 7]. These studies have suggested that in the proximal tubules, megalin functionally interacts with and mediates the internalization of the other large endocytic receptor cubulin. Megalin/cubulin-mediated reabsorption has been reported for a diverse panel of ligands, including vitamin carriers, hormones and lipoproteins (For review see reference [5]), and is believed to play an important role in maintaining homeostasis and metabolism of vital substances filtered by the glomerulus. Besides megalin, cubulin also interacts with amnionless, another transmembrane protein. The two receptor proteins have been shown to share an interdependent relationship for post-translational processing and membrane trafficking [7–11]. This is evident from the intracellular retention of cubulin or amnionless in amnionless-deficient mice [9]/dogs [10] and cubulin-deficient mice [7]/patients [11], respectively.

The mechanism responsible for human protein reabsorption has so far not been established in detail. We therefore aimed to investigate the proximal tubular function in patients with megalin deficiency through analyses of renal tissue and urinary proteins.

MATERIALS AND METHODS

Patients

The study was performed according to the Declaration of Helsinki and was approved by the Regional Ethical Review Board, Uppsala, Sweden. Informed consent was obtained from all the participants including parents and daughters.

The investigations were conducted in a Swedish family encompassing two affected children presenting with classical DB/FOAR manifestations (see supplementary information for detailed patient history). Their parents were unrelated and of northern European descent, but originated from the same small community. Persistent, non-nephrotic proteinuria was identified in the elder sister from age 2 months. From the age of seven, increasing plasma creatinine (Supplementary data, Diagram 2) and urea were observed. Hereditary nephropathy was suspected but a renal biopsy at age 8 years showed minimal changes. At the age of 18 years, focal sclerosis was found in one out of seven glomeruli indicating a slowly progressive glomerularsclerosis. Low-grade proteinuria was also present from an early age in the younger sister. Her plasma creatinine and urea were raised above normal from the age of 16 and 19, respectively (Supplementary data, Diagram 2). In addition, a male sibling was born of the same parents at 38 weeks of gestation. Bilateral diaphragmatic hernias were found and despite surgical correction the child died of respiratory failure after a few hours. In addition to diaphragmatic hernias, the subsequent autopsy revealed hypo-plastic lungs, a small ventricular septum defect but no cerebral malformation. Renal material was collected for diagnostic purposes but no DNA was isolated for genetic testing.

Mutation analysis

Nucleotides are numbered according to accession number NM_004525.2 (LRP2) with +1 corresponding to the A of the ATG translation initiation codon. Genomic DNA was isolated from blood samples obtained from both patients and parents. LRP2 exons were amplified using standard PCR procedures with previously published intronic primers [3]. A panel of commercially available human random DNA controls from non-related UK Caucasians (186 alleles; Health Protection Agency Culture Collections, Salisbury, UK) was used to assess the pathogenicity of identified variations. Direct sequencing was performed using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Naerum, Denmark). An ABI 3130 XL Genetic Analyzer and the SeqScape® Software version 2.5 (Applied Biosystems) were used for sequence analysis. Mutation nomenclature follows the guidelines of the Human Genome Variation Society. In silico splicing prediction analysis was performed using the NNSPLICE server (0.9 version; http://fruitfly.org/seq_tools/splice.html). No additional biopsy material or cells were available for analyses of LRP2 splicing.

Analysis of urinary protein excretion

Spot urine samples collected from the patients were frozen after addition of a protease inhibitor cocktail (Complete; Roche, Hvidovre, Denmark) and stored at −80°C. Urinary protein excretion was evaluated and compared with urinary protein excretion in 20 young and healthy individuals. Urinary protein excretion was normalized using creatinine concentrations and evaluated by immunoblotting. Proteins were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to an Immobilon™-FL PVDF transfer membrane (Millipore, Copenhagen, Denmark), using the iBlot™ Dry Blotting System (Invitrogen, Taastrup, Denmark). Membranes were subsequently blocked and incubated with primary and
fluorophore-coupled secondary antibodies as per the manufacturer’s instructions (LI-COR Biosciences). Proteins were detected using the Odyssey™ infrared imager (LI-COR Biosciences, Cambridge, UK) and images were converted to grey scale using Adobe Photoshop. Urinary albumin was measured using the immunoturbidmetric Vitros 5,1 FS assay in a certified biochemical laboratory (Department of Clinical Biochemistry, Aarhus University Hospital).

**Immunohistochemistry**

Renal tissue samples collected from the patients for diagnostic purposes were fixed and embedded in paraffin for routine pathology. For light microscope immunohistochemistry, sections from the female patient kidney biopsy, deceased son as well as normal human renal cortex were prepared as previously described [12]. The sections were incubated with a primary antibody in 0.01 M phosphate-buffered saline, 0.1% bovine serum albumin and 0.02 M NaN3, followed by incubation with horse-radish peroxidase (HRP)-conjugated secondary antibody. Peroxidase labelling was visualized by incubation with diaminobenzidine and 0.03% H2O2 for 10 min. The sections were counterstained with Meier’s haematoxylin stain and examined in a Leica DMR microscope equipped with a Leica DFC320 camera. The images were transferred by a Leica TFC Twain 6.1.0 program and processed using Adobe Photoshop 8.0.

**Antibodies**

*(Primary) Rabbit anti-vitamin D-binding protein (A0021), rabbit anti-transferrin (A0061), rabbit anti-apolipoprotein A-I (apo A-I; Q0496), rabbit anti-albumin (A0001), rabbit anti-α1-microglobulin (α1-M; Q0495), rabbit anti-retinol-binding protein (RBP; A0040) and rabbit anti-β2-microglobulin (β2-M; A0072) were all polyclonal anti-human antibodies (Dako, Transduction Laboratories™, Franklin Lakes, NJ), rabbit anti-human amnionless [8] rabbit anti-rat cubilin [13] and rabbit anti-human megalin [14] (Kindly provided by Dr S.K. Moestrup). *(Secondary) IRDye™—(LI-COR), Alexa Fluor™—(Invitrogen) and HRP-conjugated (Dako).*

**RESULTS**

**Molecular analyses of LRP2**

Direct sequencing of the LRP2 gene revealed a novel homozygous G → A transition (c.2639 + 1G > A) located at the fully conserved donor splice site of intron 18 (Supplementary data, Figure S1B) in the two affected children presenting with classical DB/FOAR manifestations including hearing impairment, high-grade myopia and low-molecular-weight proteinuria (see supplementary information for detailed patient history). Both parents were heterozygous at this position consistent with the autosomal recessive inheritance of DB/FOAR. No guanine to adenine exchange was detected in 186 ethnically matched control alleles supporting that this is a pathogenic mutation of the LRP2 gene. In silico analysis showed no recognition of the mutated donor splice site. Consequently, this mutation most likely results in aberrant splicing and megalin dysfunction or in functional LRP2 null alleles consistent with the clinical diagnosis of DB/FOAR.

**Renal consequences of megalin deficiency**

No major morphological abnormalities were observed at a light microscopic level in a renal biopsy from the older daughter at the age of 8 years (Supplementary data, Figure 2B, D and F) showing that megalin is not crucial for human kidney organogenesis. Interestingly, however, at the age of 18 years, minor glomerular changes consistent with focal glomerulosclerosis were present. Concordantly, both patients had elevated plasma creatinine values and a reduced estimated glomerular filtration rate (eGFR) at the age of 20 years using both the Modification of Diet in Renal Disease and cystatin C-based formulas (see Supplementary data, Diagram 2).

Consistent with molecular analyses, immunoreactive megalin was identified in neither the renal biopsy, obtained from the older daughter (Figure 1A and B), nor in the post-mortem kidney cortex from the deceased boy (not shown). Also, no megalin was detected in urine from both affected sisters (Figure 2A). No endocytic uptake of the established megalin ligand retinol-binding protein (RBP) was observed in proximal tubules either (Supplementary data, Figure 2A and B). Consistent with reduced proximal tubular uptake, increased urinary excretion of RBP was identified in both female patients (Figure 2B).

Immunostaining for cubilin and amnionless showed normal apical localization in the renal biopsy sample and autopsy material (Figure 1C and D and not shown) showing that cubilin in the human proximal tubular epithelium is apically sorted in the absence of functional megalin. Interestingly, increased urinary excretion of cubilin was observed in both patients compared with the non-affected heterozygous parents and two healthy controls (Figure 2A). Increased urinary excretion and lack of endocytic uptake were also observed for apo A-I (an established cubilin ligand) and α1-M (an established ligand of both megalin and cubilin) (Figure 2B and Supplementary data, Figure 2C–F). Increased urinary excretion of vitamin D-binding protein (VDBP) was identified in both patients (Figure 2B); however, immunostaining of the patient biopsy for VDBP revealed some endocytic uptake in a number of proximal tubules although strongly reduced (Figure 3A and B). Immunoblotting analyses of the patient urine samples furthermore revealed increased urinary excretion of additional megalin ligands, including β2-M, cathepsin b, cathepsin d as well as the cubilin ligand transferrin (Figure 2B). In contrast, immunoblotting analyses of urine from the heterozygous parents revealed no increase in the urinary excretion of the analysed proteins (data not shown). The decreased endocytic uptake of cubilin and megalin ligands observed in these patients is reflected in the absent proximal tubular clathrin staining identified in the patient renal tissue (Figure 3F). Overall, these data demonstrate that the clearance of proteins from the glomerular
filtrate in humans relies on a close functional interaction between megalin and cubilin.

**Total protein and albumin excretion**

A coomassie stain of the patient urine samples (Figure 2C) revealed a characteristic urinary protein profile with 6–7 distinct bands in line with previous observations in DB/FOAR patients [3]. Total protein excretion was 1.0 and 1.3 g/L (Figure 2C) of which albumin constituted approximately 10% (∼70 and 110 mg/L with albumin/creatinine ratios of 6.9 and 11.7 mg/mmol, respectively). As no endocytic uptake of albumin was detected in megalin-deficient proximal tubules (Figure 3C and D), these data provide a reliable estimate of human albumin filtration. Taken together, these data show that human albumin filtration is in the micro-albuminuric range.

**DISCUSSION**

In this study, we investigated the renal consequences of megalin deficiency. Direct sequencing of the megalin-encoding gene in a family with three affected children identified a novel functional LRP2 null mutation consistent with a clinical DB/FOAR diagnosis. Analyses of renal material and urine from the megalin-deficient patients showed a clearly reduced proximal tubular uptake with increased urinary excretion of filtered plasma proteins including ligands of both megalin and cubilin. Staining for clathrin, which plays a major role in formation of endocytic vesicles, identified an almost-absent apical staining in proximal tubules of the patient material. This hereby supports that proximal tubular endocytic uptake is severely reduced as previously suggested by studies from megalin-deficient mice [15]. In addition, these data show that megalin-mediated endocytosis constitutes the major part of endocytic activity in proximal tubular cells.

Immunostaining for cubilin and amnionless in the patient renal material showed a normal apical localization indicating that cubilin is expressed and independently sorted in the human kidney. This is consistent with observations from mice [7, 16] as well as recent immunohistochemical analyses of renal tissue from a cubilin-deficient patient [11]. Although cubilin and amnionless localize normally in patient proximal tubules, interestingly, we observed an increased urinary excretion of cubilin in the two patients. This may indicate a higher overall proximal tubular expression of cubilin in the megalin-deficient patients but may also reflect that cubilin is shed to the urine in the absence of megalin. Also, increased urinary excretion and no tubular endocytic uptake were observed for apo A-I (cubilin ligand) and α₁-M (shared cubilin and megalin ligand). A recent study of a renal biopsy from a cubilin-deficient patient showed a crucial role for cubilin in tubular reabsorption of filtered apo A-I [11] but not for α₁-M. Taken together, the studies suggest that in humans megalin is important for the endocytosis of cubilin-bound apo A-I [11] but not for α₁-M. Furthermore, this supports a functional interaction between the two receptors for tubular reabsorption in the human kidney.

Megalin and cubilin bind VDBP in vitro with equal affinity and both have been shown to mediate reabsorption of VDBP-bound 25-OH vitamin D, which is important for final activation of the vitamin in proximal tubular epithelial cells [17, 18]. In the megalin-deficient patients, urinary excretion of VDBP was increased; however, residual endocytic uptake
of VDBP was also identified. In addition, increased urinary excretion of VDBP has been identified in humans and dogs with cubilin dysfunction [11, 18, 19], and recently, it was also shown that tubular reabsorption of VDBP was not abolished in a cubilin-deficient patient [11]. Taken together, this shows that in humans normal and complete tubular reabsorption of VDBP depends on both megalin and cubilin. Interestingly, in mice, megalin seems to be sufficient for total clearance of VDBP as VDBP is not observed in urine from cubilin-deficient mice but only in urine from megalin-deficient mice [7]. Hence, this illustrates that the mechanism for tubular protein reabsorption may differ to some degree in mouse and man. These findings hereby show that cubilin may be able to mediate endocytosis of VDBP in the human kidney through interaction with amnionless that entails two copies of the endocytic NPXY motif [8].

Our studies have demonstrated that the underlying molecular mechanism for the very characteristic low-molecular-weight proteinuria consistently observed in DB/FOAR patients is proximal tubular dysfunction of megalin. DB/FOAR syndrome shows a limited phenotypic overlap with a number of other syndromes [2], including Pallister–Killian syndrome [OMIM #601803], Fryns syndrome [OMIM #229850], Chudley–McCullough syndrome [OMIM #604213], Acrocallosal syndrome [OMIM #200990] and Craniofrontonasal syndrome [OMIM #304110]; however, the non-renal anomalies in these patients do not resemble those found in DB/FOAR syndrome. Thus, we suggest that the characteristic facial features and functional deficits in combination with the distinct urinary protein profile consistently observed in DB/FOAR patients should allow the diagnosis of this syndrome without the need for
laborious analyses of the large \textit{LRP2} gene. Unfortunately, no increase in urinary protein excretion was observed in the heterozygous parents, demonstrating that screening for \textit{LRP2} mutation carriers through analyses of urinary protein excretion is not possible.

Since tubular uptake of most filtered proteins reabsorbed by megalin-mediated endocytosis is abolished in these patients, quantification of the urinary excretion of such proteins should provide an estimate of the amount of protein filtered in the human glomeruli. In particular, proximal tubular reabsorption of albumin in mice was recently shown to depend on simultaneous expression of cubilin and megalin \cite{7, 16}. Consistent with this, no endocytic uptake of albumin was identified in these patients, and thus, the urinary albumin excretion rate provides an estimate of the filtered amount of albumin. The total urinary albumin excretion in these patients was in the micro-albuminuric range (<200 mg/L) suggesting that <500 mg of albumin is normally filtered per day. This estimate is consistent with previous measurements from micro-puncture studies in rats \cite{20} as well as a recent study of conditional megalin and cubilin double-deficient mice \cite{16}, but orders of magnitudes lower than the values obtained using \textit{in vivo} two-photon microscopy \cite{21, 22}. Glomerular filtration of albumin is a controversial subject \cite{23}. However, our studies of the tubular handling of filtered albumin in the DB/FOAR patients as well as in megalin and cubilin double-deficient mice support that normal albumin filtration is compatible with micro-albuminuria and not in the nephrotic range (>3.5 g/day) as otherwise suggested \cite{21}.

In both affected patients a slow, progressive loss of renal function was identified, associated with histological evidence of focal glomerulosclerosis. Such changes have so far not been reported in megalin-deficient mice. Recently, megalin was identified in the human glomerulus \cite{14} and although the functional implications of glomerular expression in humans have not been investigated, this points to the possibility of megalin being important for maintaining normal glomerular function.

In conclusion, our analyses clearly demonstrate an essential role for megalin in human tubular reabsorption of several filtered proteins allowing an estimate of the normal glomerular filtration of albumin based on the urinary excretion rate in these patients. Furthermore, the characteristic urinary protein profile shown to be caused by proximal tubular
dysfunction of megalin may be utilized as a diagnostic marker for DB/FOAR.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.

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**CONFLICT OF INTEREST STATEMENT**

None declared.

**REFERENCES**


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