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CLINICAL STUDY

Urine matrix metalloproteinases and their extracellular inducer EMMPRIN in children with chronic kidney disease

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Abstract

Background: Transforming growth factor (TGF)beta1 and matrix metalloproteinases (MMPs) play an essential role in CKD-related tissue remodeling. However, there are no data on urine MMPs and their extracellular inducer EMMPRIN in CKD patients. The aim of study was to assess the concentrations of MMP-2, MMP-7, MMP-9, EMMPRIN and TGFbeta1 in serum and urine of CKD children and to analyze the potential relations between those parameters. *Methods*: Forty-one pre-dialysis CKD children and 23 age-matched controls were enrolled in the study. The concentrations of analyzed parameters were assessed by ELISA. *Results*: Serum and urine values of MMP-2, MMP-7, MMP-9, EMMPRIN and TGFbeta1 were significantly elevated in CKD patients versus controls. The MMP-2 and MMP-9 levels in urine correlated significantly with the corresponding values in serum, whereas MMP-7, EMMPRIN and TGFbeta1 urine concentrations did not. There were also significant correlations between urine values of all parameters. *Conclusions*: The increased urine levels of MMPs, EMMPRIN and TGFbeta1 indicate enhanced proteolysis and renal tissue remodeling. In the case of MMP-7, EMMPRIN and TGFbeta1 those disturbances seem independent of enhanced serum activity of the corresponding enzymes. The urine MMP-7 and EMMPRIN concentrations may serve as new independent indices of tissue remodeling and renal interstitial fibrosis in children with CKD.

Keywords

EMMPRIN, MMP-2, MMP-7, MMP-9, TGFbeta1

History

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Introduction

Renal interstitial fibrosis is the final common pathway in the chronic kidney disease (CKD) progression, independent of its origin.[1] One of the mechanisms responsible for those pathological changes is the epithelial–mesenchymal transition (EMT), triggering the transformation of tubular epithelial cells into the mesenchymal phenotype. The subsequent migration of mesenchymal cells through the extracellular matrix is followed by their transformation into the active myofibroblasts, taking part in the matrix excessive deposition and subsequent fibrosis.[2,3]

Transforming growth factor(TGF)beta1 is the master regulator of the above-mentioned processes.[4,5] Its major role of a pro-fibrotic stimulant has been proved in the *in vitro* models and clinical studies, confirming the reduction of fibrosis and injury after the administration of anti-TGFbeta antibodies.[6]

Matrix metalloproteinases (MMPs) are also known for their proteolytic activity, regulating the extracellular matrix content and tissue remodeling.[7] Thus, matrix degradation has long been considered a tool for anti-fibrotic protection. The animal studies have spoken in favor of such theory, showing that renal fibrosis is connected with the altered expressions of MMPs in the kidney.[8,9] However, recent investigation has revealed that MMPs are also able to induce the EMT and, therefore, act pro-fibrotically.[10,11] Moreover, the connection between the extracellular matrix metalloproteinase inducer (EMMPRIN) and fibrotic signaling pathways has also been discovered.[12] Therefore, MMPs probably present the functional ambiguity, depending on the environmental conditions or the stage of fibrosis progression.

The increased serum levels of MMPs have been described both in children and adults with CKD,[13–16] whereas the EMMPRIN analysis in the CKD population concerned only our previous investigation.[15,16] However, the knowledge about excretion of MMPs with urine, as well as about their potential applicability as the indicators of interstitial fibrosis in that group of patients, is limited to the experimental data and considers renal transplantation.[17,18] There are no results of such investigation in the patients with CKD—either children or adults. There are no data on the probable interplay between those parameters in serum and urine, either on the impact that EMMPRIN could have on the MMP urine excretion in CKD patients.

In the pediatric population the majority of patients develop CKD due to the anomalies within the urinary tract and kidney biopsy is not of diagnostic value in such cases. There is,

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however, constant need for noninvasive tools assessing the fibrotic processes responsible for renal failure progression.

Therefore, the aim of this study was to assess the concentrations of MMP-2, MMP-7, MMP-9, EMMPRIN and of a well-established fibrosis marker, TGFbeta1, both in serum and urine of pre-dialysis CKD children and to analyze the potential relations between those parameters.

Methods

Patient characteristics

Sixty four patients enrolled in the study were divided into two groups. Basic demographic and clinical data are shown in Table 1.

The first group consisted of 41 children with CKD stages 3–5 (17 girls, 24 boys; median age 11 years, interquartile range 4–17 years) treated conservatively (median GFR calculated according to the Schwartz formula 26 mL/min/1.73sq m.).[19] The diseases leading to CKD were: reflux nephropathy (19 cases), chronic glomerulonephritis (10), chronic pyelonephritis (6), polycystic kidney disease (4) and hemolytic uremic syndrome (2). Twenty-three children (13 girls, 10 boys, median age 10.5 years, range 5–16.5 years) with primary nocturnal enuresis and normal kidney function, served as controls.

None of the patients has shown clinical evidence of infection, had diabetes, malignancies or vasculitides, smoked, took antibiotics, statins. Nobody was treated with corticosteroids or immunosuppressive therapy for at least 12 months. The patients were also free of such co-morbidities as cardiovascular disease, peripheral vascular disease or obesity. In the CKD group 10 children were normotensive according to the criteria of the European Society of Hypertension in children and adolescents,[20] in 31 patients blood pressure was well controlled with the use of ACE inhibitors (14), calcium channel blockers (10 patients) and β-blockers (3 children), 4 patients needed combined therapy. In all CKD patients phosphate binders and vitamin D metabolites were supplemented.

Informed consent was obtained from the subjects and their parents, if necessary. The research project has been approved by the University ethics committee, in accordance with the Helsinki declaration.

Blood samples were drawn from peripheral veins after an overnight fast. Samples were clotted for 30 min, centrifuged at room temperature for 10 min, and then serum was stored at −20 °C until assayed. Urine was collected aseptically from the first morning sample, centrifuged at room temperature for 10 min and then stored at −20 °C until assayed.

Table 1. Patient characteristics.

Parameter	Median values (lower – upper quartile) of analyzed parameters	
	Control group ($n = 23$)	CKD ($n = 41$)
Age [years]	10.5 (5.0–14.5)	11.0 (4.0–17.0)
Gender	13 girls; 10 boys	17 girls; 24 boys
eGFR [mL/min]	105.0 (97.0–112.3)*	26.0 (16.8–38.0)
Urea [mg/dL]	32.0 (25.5–37.0)*	77.0 (55.0–94.5)
Albumin [g/dL]	–	4.3 (3.8–4.5)
Hemoglobin [g/dL]	12.8 (11.7–13.9)*	11.2 (10.5–12.2)
Parathormone [pg/mL]	–	125.0 (46.1–223.0)
hsCRP [mg/L]	0.5 (0.24–1.34)	0.6 (0.18–1.37)
Proteinuria [g/L]	0.01 (0–0.1)*	0.4 (0.03–0.6)
Urine creatinine [mg/dL]	114.0 (100.0–126.0)*	90.0 (72.0–110.0)

Mann–Whitney U test: *$p < 0.001$ control group versus CKD.

Assay characteristics

The serum and urine concentrations of MMP-2 (molecular mass 72kDa), MMP-7 (25 kDa), MMP-9 (82–92 kDa), EMMPRIN (35–65 kDa) and TGFβ1 (25 kDa) were evaluated by commercially available ELISA kits (MMP-2—R&D Systems (Abingdon, UK), reagent kit DMP200; MMP-7—R&D Systems, reagent kit DMP700; MMP-9—R&D Systems, reagent kit DMP900; EMMPRIN—R&D Systems, reagent kit DEMP00; TGFβ1—R&D Systems, reagent kit DB100B). Standards, serum and urine samples were transferred to 96 well microplates pre-coated with recombinant antibodies to human MMP-2, MMP-7, MMP-9, EMMPRIN and TGFβ1. Captured proteins were then detected using monoclonal antibodies against MMP-2, MMP-7, MMP-9, EMMPRIN and TGFβ1 conjugated to horseradish peroxidase. Next, the assay was developed with tetramethylbenzidine substrate and blue color was developed proportionately to the amount of captured protein. The addition of acid stop solution ended the color development and converted it to the endpoint yellow. The intensity of the latter was measured in a microplate reader at 450 nm, with the correction wavelength at 550/650 nm. Each sample was tested in duplicate and the arithmetical mean was considered a final result. Measurements were performed according to the manufacturer's instructions, results were calculated by reference to standard curves.

The intra-assay and inter-assay coefficients of variation (%CV) for examined parameters did not exceed 6.0% and 8.5%, respectively, and were as follows: MMP-2— ≤ 4.7 and ≤ 8.2, MMP-7— $\leq 4.6\%$ and $\leq 4.7\%$, MMP-9— $\leq 2.3\%$ and $\leq 7.3\%$, EMMPRIN— $\leq 4.5\%$ and $\leq 5.7\%$, TGFβ1— $\leq 6.0\%$ and $\leq 8.5\%$. Limits of detection: MMP-2—0.03 ng/mL, MMP-7—0.02 ng/mL, MMP-9—0.16 ng/mL, EMMPRIN—9.77 pg/mL, TGFβ1—4.6 pg/mL.

The serum and urine creatinine were assessed with the Creatinine (Enzymatic) OSR61204 reagent on the Beckman Coulter (Brea, CA) AU2700 analyzer. High sensitivity (hs)CRP was assessed by immunonephelometry with Siemens (Munich, Germany) CardioPhase hsCRP reagent on the BN II System analyzer.

Statistical analysis

The results are expressed as median values and interquartile ranges. Since the null hypothesis of normality of distribution was rejected by the Shapiro–Wilk test, comparisons in pairs were evaluated by using nonparametric tests (Mann–Whitney U). Relations between parameters were defined by Spearman's correlation coefficient R. The statistically significant correlations were then analyzed by linear regression analysis in CKD patients. The linear regression equations were calculated as $y = \beta x + a$ (y—dependent variable, β—regression coefficient, x—independent variable, a—constant term). A p value < 0.05 was considered significant.

Results

MMPs, EMMPRIN and TGFbeta1

The serum MMP-2, MMP-7, MMP-9, EMMPRIN and TGFbeta1 concentrations in CKD children were significantly higher versus controls (Table 2). The further analysis revealed no significant differences between the patients with glomerulopathies and those with congenital anomalies of the kidney and urinary tract (CAKUT).

The urine levels of all MMPs, EMMPRIN and TGFbeta1, normalized to urine creatinine, were also significantly increased when compared to the control group (Table 3). Likewise in the serum, the comparison of parameters between the children with CKD in the course of chronic glomerulonephritis and those with CKD due to anomalies in the urinary tract has shown no statistically significant differences.

Correlations and regression analysis

The MMP-2 and MMP-9 levels in urine correlated significantly with the corresponding values in serum, whereas MMP-7, EMMPRIN and TGFbeta1 urine concentrations did not (Table 4). However, the strongest correlations were observed between the urine concentrations of analyzed parameters (R values varied from 0.63 to 0.96; p value was always <0.0000001). Moreover, urinary MMP-7 and MMP-9 were good predictors of both urinary TGFbeta1 ($R^2=0.92$, $p<0.0001$; $R^2=0.93$, $p<0.0001$; respectively) and urinary EMMPRIN ($R^2=0.91$, $p<0.0001$; $R^2=0.80$, $p<0.003$; respectively). The urinary EMMPRIN predicted well the urinary TGFbeta1 ($R^2=0.87$, $p<0.0001$).

None of the analyzed parameters correlated with eGFR. None of them were related to hsCRP or other biochemical markers characteristic for CKD (mentioned in Table 1) either.

Discussion

Our investigation has revealed the significantly increased concentrations of all examined metalloproteinases, their inducer EMMPRIN and TGFbeta1, both in serum and in urine of children with chronic kidney disease, when compared to the controls.

The MMP-2 and MMP-9 roles in matrix remodeling have been studied extensively on animal models, confirming the increased MMP-2 and decreased MMP-9 expressions in fibrotic kidneys.[8,9] The studies in humans with CKD concerned mainly adults, showing either increased or decreased concentrations in pre-dialysis and hemodialyzed patients.[13,14] Our preliminary investigation has revealed elevated serum MMPs in children during both pre-dialysis and dialysis periods.[15,16] However, none of those analyses has examined urine concentrations of metalloproteinases or potential correlations between them and corresponding serum values. Our investigation has shown for the first time the significantly increased concentrations of MMP-2 and MMP-9 both in serum and urine of children with the advanced stages of CKD, as well as statistically significant correlations between them. The elevation of MMP-2 and MMP-9 serum concentrations may be the consequence of various processes. The accumulation due to diminishing GFR seems possible, because both gelatinases are the molecules of middle molecular mass and glomerular filtration is quite improbable, unless through the damaged filtration barrier. Another trigger may be the stimulation by pro-inflammatory cytokines or the reciprocal activation by proteolysis through MMPs and EMMPRIN.[7,21]

So far, the urine concentrations of gelatinases have been tested for their ability to predict cancer within the urinary tract, but the results were ambiguous.[22,23] In our study, controls presented with very low, when compared to the serum values, urine levels of MMP-2 and MMP-9. The serum

Table 2. The concentrations of analyzed parameters in serum of CKD children and controls.

Parameters in serum	Median values (lower – upper quartile)	
	Control group ($n=23$)	CKD ($n=41$)
MMP-2 [ng/mL]	94.53 (88.88–115.77)*	159.45 (156.05–167.92)
MMP-7 [ng/mL]	2.23 (2.17–2.91)*	2.97 (2.27–3.05)
MMP-9 [ng/mL]	94.30 (91.00–100.80)*	415.60 (397.90–427.60)
EMMPRIN [pg/mL]	871.93 (854.86–906.07)*	1175.03 (1150.55–1211.54)
TGFbeta1 [ng/mL]	1221.99 (1195.0–1242.9)*	1738.88 (1717.61–1760.18)

Mann–Whitney U test: *$p<0.0001$ control group versus CKD.

Table 3. The concentrations of analyzed parameters in urine of CKD children and controls.

Parameters in urine	Median values (lower – upper quartile)	
	Control group ($n=23$)	CKD ($n=41$)
MMP-2 [ng/mg creat]	1.19 (1.03–1.43)*	2.64 (1.92–3.87)
MMP-7 [ng/mg creat]	0.009 (0.008–0.10)*	3.11 (2.71–3.91)
MMP-9 [ng/mg creat]	1.84 (1.63–2.12)*	4.49 (3.95–5.25)
EMMPRIN [pg/mg creat]	375.02 (313.30–402.44)*	1082.36 (1938.19–1361.49)
TGFbeta1 [ng/mg creat]	42.55 (38.44–47.97)*	258.55 (240.49–323.66)

Mann–Whitney U test: *$p<0.0001$ control group versus CKD

Table 4. Correlations between examined parameters in serum and urine of CKD children.

Parameter	Serum MMP-2 [ng/mL]	Serum MMP-7 [ng/mL]	Serum MMP-9 [ng/mL]	Serum EMMPRIN [pg/mL]	Serum TGFbeta1 [ng/mL]
Urine MMP-2	$p = 0.00006$	$p = 0.0003$	$p = 0.00003$	$p = 0.41$	$p = 0.50$
[ng/mg creat]	$R = -0.59$	$R = 0.53$	$R = 0.61$	$R = 0.13$	$R = -0.11$
Urine MMP-7	$p = 0.31$	$p = 0.9$	$p = 0.44$	$p = 0.72$	$p = 0.84$
[ng/mg creat]	$R = -0.16$	$R = -0.02$	$R = 0.13$	R = 0.06	$R = -0.03$
Urine MMP-9	$p = 0.039$	$p = 0.16$	$p = 0.048$	$p = 0.21$	$p = 0.76$
[ng/mg creat]	$R = -0.32$	$R = 0.22$	$R = 0.31$	$R = 0.19$	$R = -0.05$
Urine EMMPRIN	$p = 0.49$	$p = 0.94$	$p = 0.64$	$p = 0.69$	$p = 0.91$
[pg/mg creat]	$R = -0.11$	$R = -0.01$	$R = 0.08$	$R = -0.06$	$R = 0.02$
Urine TGFbeta1	$p = 0.32$	$p = 0.87$	$p = 0.29$	$p = 0.03$	$p = 0.71$
[ng/mg creat]	$R = -0.16$	$R = 0.03$	$R = 0.17$	$R = 0.27$	$R = -0.06$

R – Spearman's correlation coefficient.

and urine concentrations in CKD children have risen proportionately, with urine values still incomparably low to the corresponding serum values. Such situation could result mainly from the leakage of serum gelatinases through destroyed filtration barrier at the late stages of CKD. Strong correlations between urine and serum levels of MMP-2 and MMP-9 seem to confirm that hypothesis. Additionally, the significant correlations between urine MMP-9 and TGFbeta1, strengthened by their predictive value, seem to confirm the engagement of gelatinase B in renal fibrosis and the utility of its urine concentrations in assessing CKD-related processes.

Different conditions seemed to determine the serum and urine concentrations of MMP-7 and EMMPRIN. Both of them, similarly to gelatinases, are able to cleave a soluble form of one another and reciprocally, which could be one of the explanations of their serum increment in the course of CKD.[21] Another possibility is the CKD-related accumulation along with decreasing GFR.

So far, the studies analyzing engagement of MMP-7 and EMMPRIN in fibrosis have been restricted to experimental models showing their pro-fibrotic activity.[12,17] The aspect distinguishing MMP-7 from other MMPs is its epithelial expression, including that within the urinary tract.[24] Such localization might explain why urinary MMP-7 is considered a useful marker of bladder cancer.[25] The same features have been attributed to EMMPRIN.[26] The intriguing fact in our investigation was that, in none of those markers, the relations between serum and urine levels were preserved, suggesting that the latter may be considered a tissue-specific production within the kidney.

The MMP-7 urine concentrations were the most evident proof, since in healthy subjects matrilysin has not been found in urine, although its low molecular weight could well justify the possibility of glomerular filtration, subsequent tubular reabsorption and degradation in physiological conditions. On the contrary, in CKD children MMP-7 values in urine have risen over 300-fold and became higher than those in serum. Thus, the presence of MMP-7 in urine of CKD patients is definitely a sign of kidney-specific pathology and thus, it could serve as an index of CKD-related anomalies, including damage to filtration barrier, renal fibrosis progression and insufficient reabsorption by proximal tubules. Another strong point adding to that theory was the fact that urine MMP-7 correlated with TGFbeta1 and predicted it sufficiently well. Similar MMP-7 attitude was observed towards EMMPRIN.

Moreover, EMMPRIN was the only parameter found at substantial concentrations in the urine of controls and, at significantly higher levels, in the urine of CKD children. Meanwhile, the values of urine concentrations in controls were three times lower than the ones in serum, whereas in CKD patients' urine and serum values were comparable. Thus, the rise of urine concentrations was more dynamic than the increase in serum levels. Such discrepancy speaks in favor of a mixed mechanism responsible for high urine concentrations of EMMPRIN, with the preponderance of kidney-specific production over the enhanced renal clearance or the insufficient tubular reabsorption. Meanwhile, the significant predictive value towards TGFbeta1 adds to the value of EMMPRIN as a parameter assessing the fibrosis-related processes. Thus, EMMPRIN becomes also a candidate marker of matrix remodeling and renal interstitial fibrosis.

Pediatric CKD-related fibrosis is still a challenge from a diagnostic point of view. The substantial amount of congenital anomalies within the urinary tract, leading to chronic kidney disease, and a relatively small population, in comparison to adult CKD, requires constant search for non-invasive tools to follow up the progression of the disease. Hopefully, the above mentioned urine markers may serve as new indices of fibrosis-related processes in the late stages of CKD, when kidney biopsy is already an unavailable procedure.

Conclusions

The increased urine levels of examined MMPs, EMMPRIN and TGFbeta1 may indicate enhanced proteolytic processes and renal tissue remodeling. The rise of MMP-7, EMMPRIN and TGFbeta1 concentrations in urine seemed independent of the enhanced serum activity of the corresponding enzymes, contrarily to MMP-2 and MMP-9. Therefore, urinary MMP-7 and EMMPRIN concentrations may serve as new independent indices of tissue remodeling and renal interstitial fibrosis in the course of CKD in the pediatric population.

Declaration of interest

The authors report no conflicts of interest.

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