FRACTIONAL EXCRETION OF SURVIVIN, EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER, AND MATRIX METALLOPROTEINASE 7 IN CHILDREN WITH CHRONIC KIDNEY DISEASE

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ABSTRACT

Background: Epithelial–mesenchymal transition (EMT) is defined as a transformation of tubular epithelial cells into mesenchymal ones. These cells migrate through the extracellular matrix and change into active myofibroblasts, which are responsible for excessive matrix deposition. Such changes may lead to tubular dysfunction and fibrosis of the renal parenchyma, characteristic of chronic kidney disease (CKD). However, there are no data on potential EMT markers in children with CKD. The aim of our study was to assess the usefulness of fractional excretion (FE) of survivin, E-cadherin, extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinase (MMP)7, and transforming growth factor beta 1 (TGF-β1) as potential markers of CKD-related complications such as tubular damage and fibrosis.
Methods: Forty-one pre-dialysis children with CKD Stages 3–5 and 23 age-matched controls were enrolled in the study. The serum and urine concentrations of analysed parameters were assessed by an enzyme-linked immunosorbent assay test.
Results: Tubular reabsorption of all analysed parameters was >99% in the control group. All FE values rose significantly in children with CKD, yet they remained <1% in the case of E-cadherin and TGF-β1. The highest FE values in CKD children were those of survivin, EMMPRIN, and MMP7: >1%.
Conclusions: FE of the examined markers may become a useful tool in the assessment of tubular dysfunction during the course of CKD. The FE of survivin, EMMPRIN, and MMP7 warrant further research as potential independent markers of kidney-specific EMT.

Keywords: Epithelial–mesenchymal transition (EMT), fibrosis, tubular damage, E-cadherin, transforming growth factor beta 1 (TGF-β1).

INTRODUCTION

Renal interstitial fibrosis is the final common pathway in chronic kidney disease (CKD), irrespective of its primary cause.[1,2] The origin of myofibroblasts, which play a pivotal role in renal fibrogenesis, is under debate.[3,4] Despite conflicting evidence, the epithelial–mesenchymal transition (EMT) is still considered one of the possible mechanisms responsible for the appearance of fibrotic changes.[5-7] During EMT, epithelial cells lose their epithelial characteristics and gain mesenchymal features.[2,5-7] The newly formed mesenchymal cells migrate through the extracellular matrix and change into the active myofibroblasts, responsible for excessive matrix deposition and the subsequent fibrosis progression.[1,2,6,7] The ongoing discussion suggests that this process may be reversible, and could be a sign of immense kidney cell plasticity, enabling regression of fibrosis.[8,9]

Transforming growth factor beta 1 (TGF-β1) is the main EMT player and the master regulator of fibrosis, triggering early hypertrophy, apoptosis, and the atrophy of tubular epithelial cells and their transdifferentiation in order to gain the phenotype characteristic of matrix-producing myofibroblasts.[10,11] The *in vitro* models and clinical studies have

proven the essential role of TGF-β1 in fibrosis, since the administration of anti-TGF-β antibodies has resulted in the reduction of renal injury and fibrosis.[12] However, recent data show that TGF-β1 alone is responsible for a reversible transition of tubular cells, whereas additional exposure to Type I collagen, the most abundant extracellular matrix protein in renal interstitium, makes this transdifferentiation complete.[13] EMT is thus one of the trigger factors in fibrosis, whereas the irreversible parenchymal damage is a finely tuned process, strengthened by the *in situ* conditions.

The limits of matrix metalloproteinase (MMP) engagement in EMT and renal fibrosis have primarily been drawn as far as the regulation of extracellular matrix content and tissue remodelling. However, recent studies have revealed that the MMP influence may be more significant as they can have pro-fibrotic functions through EMT induction.[14,15] MMP7 occupies a unique position among metalloproteinases. It promotes renal fibrosis through its proteolytical activity in two ways. Firstly, it triggers EMT by shedding E-cadherin, and then aggravates apoptosis through Fas ligand cleavage.[16] Moreover, urinary MMP7 is a biomarker of the pro-fibrotic Wnt/β-catenin activity in the kidney.[17]

The extracellular matrix metalloproteinase inducer (EMMPRIN) also interacts with the fibrotic signalling pathways.[18] Such correlation has been confirmed by our preliminary study concerning children with CKD.[19] E-cadherin is an adhesion molecule released into the circulation as a consequence of the cell-cell detachment.[20-22] Anoikis, a specific form of apoptosis, enables the elimination of these cells thus preventing the reattachment in an inappropriate location and the formation of metastasis.[20,22] The loss of E-cadherin expression, resulting in the molecule accumulation in serum, is a hallmark of EMT, strictly connected with the resistance to anoikis.[20,22] It has scarcely been analysed in the patients with CKD.[23] Survivin is another protein acting in an anti-apoptotic way, enabling the rescue from anoikis through the activation of nuclear factor kappa B.[24] Recent interest has transformed the potential nephrological engagement of survivin, revealing the paramount importance of its expression in mice undergoing the renal proximal tubule recovery after acute kidney damage.[25] However, the role of survivin has not yet been assessed in patients with CKD except for in our preliminary results concerning children.[23]

The idea of analysing the fractional excretion (FE) of various parameters as a substitute of tubular dysfunction is novel in CKD patients and it has been historically used for assessing phosphate metabolism.[26] The aim of this study was therefore to analyse both known and new molecules engaged in EMT, resulting in tubular dysfunction and subsequent fibrosis. We have assessed the FE of survivin, E-cadherin, EMMPRIN, active MMP7, and TGF-β1 in children with CKD Stages 3–5 and in a control group. We have also analysed the potential relations between these parameters and their applicability as markers of CKD-related phenomena such as tubular damage and fibrosis.

METHODS

Patient Characteristics

Sixty-four patients enrolled in the study were divided into two groups. The basic demographic and biochemical data are given 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 glomerular filtration rate of 26 mL/min/1.73 m^2, calculated according to the Schwartz formula).[27] The diseases leading to CKD were: reflux nephropathy (n=19), chronic glomerulonephritis (n=10), chronic pyelonephritis (n=6), polycystic kidney disease (n=4), and haemolytic uraemic syndrome (n=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 had clinical evidence of infection, diabetes, malignancies, or vasculitis, smoked, or took antibiotics or statins. None of the patients had been treated with corticosteroids or immunosuppressive therapy for at least 12 months. The patients were also free of such comorbidities 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 (ESH) in children and adolescents.[28] In 31 patients with CKD, blood pressure was well controlled with the use of angiotensin converting enzyme inhibitors (14 children), calcium channel blockers (10 patients), or beta blockers (3 children); 4 patients needed combined therapy. In all CKD patients, phosphate binders and vitamin D metabolites were supplemented.

Table 1: Patient characteristics.

Parameter	Median values (Interquartile range)	
	Control group (n=23)	CKD (n=41)
Age (years)	10.5 (5.0–14.5)	11.0 (4.0–17.0)
Gender	13 female, 10 male	17 female, 24 male
eGFR (mL/min/1.73m^2)	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)	NA	4.3 (3.8–4.5)
Haemoglobin (g/dL)	12.8 (11.7–13.9)*	11.2(10.5–12.2)
Parathormone (pg/mL)	NA	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 (0.0–0.1)*	0.4 (0.03–0.6)

*Mann–Whitney U test: p<0.001 control group versus CKD.
CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; hsCRP: high sensitivity C-reactive protein; NA: not assessed.

$$\frac{(\text{Parameter urine concentration})\times(\text{serum creatinine concentration})}{(\text{Parameter serum concentration})\times(\text{urine creatinine concentration})} \times 100$$

Equation 1: Calculation for fractional parameter excretion.

Informed consent was obtained from the subjects, and their parents if necessary. The research project was 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 minutes, centrifuged at room temperature for 10 minutes, 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 minutes, and then stored at -20°C until assayed.

Assay Characteristics

The serum and urine concentrations of survivin (molecular mass 16.5 kDa), E-cadherin (120 kDa), EMMPRIN (35–65 kDa), MMP7 (25 kDa), and TGF-β1 (25 kDa) were evaluated by the commercially available Quantikine® Human Immunoassay ELISA (enzyme-linked immunosorbent assay) kits (survivin: R&D Systems, reagent kit DSV00; E-cadherin: R&D Systems, reagent kit DCADE0; EMMPRIN: R&D Systems, reagent kit DEMP00; MMP7: R&D Systems, reagent kit DMP700; TGF-β1: R&D Systems, reagent kit DB100B). Standards, serum, and urine samples were transferred to 96-well microplates pre-coated with recombinant antibodies to human survivin, E-cadherin, EMMPRIN, MMP7, and TGF-β1. Measurements were performed according to the manufacturer's instructions and results were calculated by reference to standard curves.

The serum and urine creatinine were assessed with the Beckman Coulter® Creatinine (enzymatic) OSR61204 Reagent on the AU2700 Chemistry Analyzer™. High sensitivity C-reactive protein was assessed by immunonephelometry, with Siemens CardioPhase® high sensitivity C-reactive protein reagent, on the BN™ II System analyser. The fractional parameter excretion was calculated according to the formula in Equation 1.

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 Pearson's correlation coefficient r and additionally pictured by cluster analysis.

Table 2: The serum and urine concentrations, and fractional excretion values of analysed parameters in children with chronic kidney disease and controls.

Analysed Parameters	Median values (Interquartile range)	
	Control group (n=23)	CKD (n=41)
Serum survivin (ng/mL)	44.40 (40.42–47.97)*	98.51 (88.19–107.13)
Serum E-cadherin (ng/mL)	31.45 (30.45–32.68)*	98.50 96.34–103.58)
Serum EMMPRIN (pg/mL)	871.93 (854.86–906.07)*	1175.03 (1150.55–1211.54)
Serum MMP7 (ng/mL)	2.23 (2.17–2.91)*	2.97 (2.27–3.05)
Serum TGF-β1 (ng/mL)	1221.99 (1195.0–1242.9)*	1738.88 (1717.61–1760.18)
Serum creatinine (mg/mL)	0.69 (0.64–0.76)*	2.55 (1.3–3.7)
Urine survivin (ng/mL)	41.49 (38.21–45.42)*	86.50 (80.85–93.84)
Urine E-cadherin (ng/mL)	3.34 (3.17–3.42)*	6.54 (6.30–6.67)
Urine EMMPRIN (pg/mL)	394.39 (375.02–413.56)*	800.42 (767.25–849.63)
Urine MMP7 (ng/mL)	1.05 (1.02–1.11)*	2.36 (2.29–2.38)
Urine TGF-β1 (ng/mL)	48.37 (45.96–49.98)*	195.10 (192.39–199.62)
Urine creatinine (mg/dL)	114.00 (100.00–126.00)*	74.97 (60.0–82.0)
FE survivin (%)	0.73 (0.58–0.75)*	1.99 (1.46–3.30)
FE E-cadherin (%)	0.07 (0.06–0.07)*	0.16 (0.10–0.28)
FE EMMPRIN (%)	0.31 (0.28–0.31)*	1.56 (1.07–2.86)
FE MMP7 (%)	0.25 (0.23–0.29)*	1.89 (1.24–4.16)
FE TG-Fβ1 (%)	0.02 (0.02–0.03)*	0.29 (0.17–0.55)

*Mann-Whitney U test: p<0.0001 control group versus CKD.
FE: fractional excretion; CKD: chronic kidney disease; EMMPRIN: extracellular matrix metalloproteinase inducer; MMP7: matrix metalloproteinase 7; TGF-β1: transforming growth factor beta 1.

Statistical analysis was performed using the package Statistica 10.0 (StatSoft). A p-value <0.05 was considered significant.

RESULTS

Fractional Urinary Excretion of Survivin, E-cadherin, EMMPRIN, MMP7, and TGF-β1

The median serum and urine concentrations of all examined parameters were elevated in CKD patients versus controls (Table 2). The values of urinary FE in all cases were significantly elevated in the CKD children when compared with the control group (Table 2). In particular, the FE values of E-cadherin and TGF-β1 were <1% in both the control and CKD groups, whereas FE for survivin, EMMPRIN, and MMP7 elevated, exceeding 1% in the CKD children (Table 2).

Correlations and Cluster Analysis

All FE values correlated significantly with each other (r>0.96; p<0.000001). Additionally, cluster analysis revealed the similarity of features pictured by EMMPRIN, E-cadherin, and TGF-β1, suggesting that their efficiency as markers is comparable and choosing one out of three would be enough to get the required information (Figure 1). None of the analysed parameters correlated with analysed biochemical markers or proteinuria.

DISCUSSION

We have shown that FE values of survivin, E-cadherin, EMMPRIN, MMP7, and TGF-β1 increase in children with CKD, when compared with controls. In our previous studies, we looked into the urine excretion of the above mentioned parameters and their correlations to the serum concentration.[19,23] The increasing urine concentrations of survivin, E-cadherin, EMMPRIN, MMP7, and TGF-β1 have turned out to be useful markers of EMT and other CKD-related complications such as fibrosis or apoptosis. Moreover, some of the analysed urine parameters were independent of serum

concentrations, suggesting that the increase of urine concentration of survivin, EMMPRIN, MMP7, and TGF-β1 in CKD is more due to the kidney production than to the parameter leakage by the damaged filtration barrier. However, those results were not sufficient to prove the origin of parameters found in urine. Therefore, we decided to verify the credibility of our hypothesis with the usage of FE.

FE has previously found its way to clinical practice in the case of sodium, enabling differentiation between pre-renal acute kidney injury and renal acute kidney injury due to tubular damage. The latter is characterised by increased FE of sodium. The data on the practical use of FE in the population with CKD is restricted to phosphate metabolism.[26] There are also single studies assessing FE of endothelin 1 or heat shock protein 27 in the context of lupus nephritis and CKD.[29,30] However, none of them dealt with a whole group of parameters characterising a particular process. Our focus was on markers picturing the variety of processes leading to tubular damage and fibrosis, which are characteristic features of CKD.

In our study, the FE of all analysed parameters was <1% in the control group, showing that in normal conditions survivin, E-cadherin, EMMPRIN, MMP7, and TGF-β1 are reabsorbed in over 99% by tubular cells. In children with CKD, FE values were significantly higher than in controls, confirming the tubular dysfunction characteristic of CKD. There was however a clear distinction between the examined markers. E-cadherin and TGF-β1 FE values remained <1% in CKD children, whereas survivin, EMMPRIN, and MMP7 FE values exceeded that border. Such an increase is suggestive of renal production of survivin, EMMPRIN, and MMP7, resulting from tubular loss and probably facilitating EMT with subsequent fibrosis and irreversible loss of renal function.

The significance of increased FE values strongly depends on the analysed parameter. Survivin is known for its anti-apoptotic activity and the connections between urine survivin and apoptotic markers have been shown in our previous study.[23]

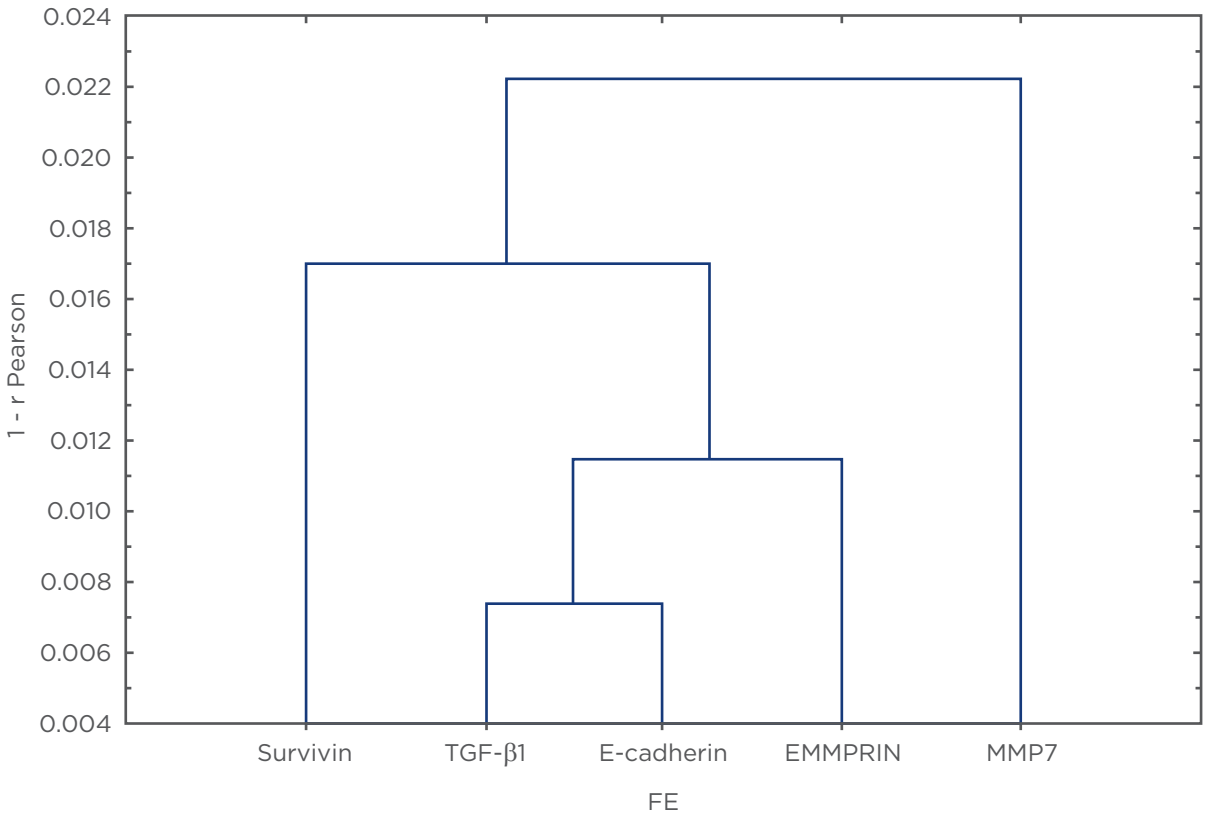

Figure 1: The cluster analysis of fractional excretion values of examined parameters in children with chronic kidney disease.

FE: fractional excretion; TGF-β1: transforming growth factor beta 1; EMMPRIN: extracellular matrix metalloproteinase inducer; MMP7: matrix metalloproteinase 7.

The essential role for survivin in tubular damage recovery after acute kidney injury has also been discovered.[25] Therefore, the increase of FE survivin value in CKD children may mean the protective reaction against aggravated apoptosis in the milieu of renal parenchyma, including damaged tubular cells. The FE survivin values may be treated as an early marker of tubular damage in the course of CKD.

The tight relations in the area of pro-fibrotic activity and EMT triggering, seen between MMPs and their EMMPRIN, have also been known for a while.[15,18] Our previous investigation has confirmed the usefulness of those parameters in assessing CKD-related complications like fibrosis.[19] It has now been shown that both EMMPRIN and MMP7 in urine might originate from the renal parenchyma, giving insight into the actual fibrotic activity of the tissue and, indirectly, the progression of kidney dysfunction. This example is even more convincing in the case of MMP7, because its urine concentration in controls was nearly negligible; the values observed in the urine of CKD children from that perspective seemed overwhelming.[19] Thus, both EMMPRIN and MMP7 FE values >1% in CKD children may indicate the tubular damage characteristic of renal failure progression.

The correlation analysis has shown significant correlations between all analysed FE values, yet the cluster analysis allowed us to eliminate the markers that described similar features. Out of three parameters (FE values of E-cadherin, TGF-β1, and EMMPRIN) we have chosen EMMPRIN FE values >1%, thus forming the homogenous marker group with FE of survivin and FE of MMP7. Our preliminary data have shown the potential usefulness of FE of survivin, EMMPRIN, and MMP7, mainly in assessing the tubular dysfunction characteristic of CKD progression. The use of these parameters as markers of the kidney-specific EMT, however promising, requires further investigation.

There are a number of limitations to this study that have to be acknowledged. The lack of comparative data and reference values for some of the examined parameters, both in adult and paediatric patients, necessitates caution in drawing conclusions. Additionally, there ought to be careful interpretation of the results due to the potential bias of a small study group and transversal design. However, the number of analysed patients and the study design were both conditioned by the overall size of the paediatric population with CKD.

CONCLUSIONS

FE values of the examined markers may become a useful tool in assessment of tubular dysfunction in the course of CKD. FE values of survivin, EMMPRIN, and MMP7 warrant further research as potential independent markers of the kidney-specific EMT.

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