The expression of cytoskeletal proteins in kidney specimens of children with primary focal segmental glomerulosclerosis

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ABSTRACT

Several studies have evaluated cytoskeletal proteins as prognostic factors for some types of nephrotic syndrome. However, studies concerning children with FSGS are scarce. This study was done to evaluate the glomerular, tubular, and interstitial expression of vimentin, desmin, and alpha smooth muscle actin (α-SMA) in kidney specimens of children with FSGS. Clinical and histologic data of 31 children with FSGS were reviewed. Thirty one formalin-fixed, paraffin-embedded kidney biopsy sections (3 μm) were selected for immunohistochemical staining. Double immunohistochemistry using a microwave-based two-color staining was applied. The mean age at onset in male and female was 56.3 ± 41.4 and 78.0 ± 60.4 months, respectively. The duration of follow-up was 46.3 ± 56.5 months. Interstitial fibrosis and tubular atrophy were reported in 42% and 54% of the patients, respectively. The latest evaluated mean blood pressure was significantly correlated with the expression of both vimentin and α-SMA in the interstitium ($P < 0.05$). However, we were not able to demonstrate any cytoskeletal protein expression as an independent predictor for renal survival. Further studies with larger sample size and longer follow-up periods are warranted to investigate the prognostic values of other histopathologic features in pediatrics with FSGS.

Key words: Alpha smooth muscle actin, desmin, focal segmental glomerulosclerosi, children, prognostic factor, vimentin

Introduction

Primary focal segmental glomerulosclerosis (FSGS) is a clinicopathologic diagnosis that manifests as proteinuria which is mostly within the nephrotic range.[1]

The term "focal" means some of the glomeruli are affected whereas the others function normally and "segmental" refers to segmental involvement of the affected glomeruli with fusion of podocytes foot processes.[2]

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This disease accounts for 7-20% of glomerular lesions in children.[3] Up to 70% of patients with FSGS have been reported to be resistant to steroids.[4] More than half of the patients have been reported to progress to end stage renal disease (ESRD) after 10 years of follow up.[5,6] In addition, this disease may recur in up to 30% of the transplanted kidneys, resulting in renal failure.[7]

Although patients with primary FSGS are generally believed to have a poor prognosis and progress to renal impairment, some enter complete remission after therapy for this disease. However, the renal survival in non-responders is worse than the responders.[8]

In the recent years, several studies have suggested certain clinical, pathologic, and immunohistochemical features as predictors for progression this disease.

Age, collapsing, and cellular histologic variants, the extent of interstitial fibrosis, blood pressure and serum creatinine concentration at the time of presentation, and also the patients' ethnicity have been reported as predictive

factors for this disease.[2,8,9-15] However, the value of these prognostic factors is still debated.

Cytoskeletal proteins such as alpha smooth muscle actin (α-SMA), desmin, and vimentin are elements expressed by myofibroblasts in the kidney tissue.[16,17] These proteins have significant role in tissue proliferation and also tubule-interstitial fibrosis in the diseased kidneys.[16] Interstitial fibrosis is known to have an important role in progression to ESRD in FSGS patients.[14] Therefore, determination of the role of these proteins in predicting long-term outcome of FSGS should be considered.

Several studies have evaluated the implication of certain cytoskeletal proteins as prognostic factors for some types of nephrotic syndrome.[18-20] However, there are few published study concerning FSGS.[21] Furthermore, as far as we are aware to date, there is no report considering cytoskeletal proteins expression as prognostic factors of FSGS in pediatric setting.

The purpose of this historical cohort study was to evaluate the role of glomerular, tubular, and interstitial expression of some cytoskeletal proteins (vimentin, desmin, and α-SMA) in determining renal survival in FSGS children. Clinical and laboratory characteristics were also investigated.

Materials and Methods

Patients

This historical cohort study was carried out in Isfahan, a large central province of Iran. In the time period between November 2007 and April 2010, we consecutively recruited 31 children with primary FSGS, who were referred to pediatric nephrology wards of the Isfahan University of Medical Sciences, the main health support organization of the region. The inclusion criteria were (i) age at onset of 1-18 years (ii) definit diagnosis of primary FSGS with renal biopsy, (iii) adequate kidney tissue in paraffin-embedded specimens to prepare new slides for immunohistologic staining, and (iv) follow-up period of at least two years for each case. The histopathologic diagnosis of FSGS was based on the following criteria: (i) lesion affecting some of the glomeruli in the renal biopsy whereas others remain unaffected and (ii) the affected glomeruli having a portion that has undergone capillary collapse with obliteration of capillary lumina with or without adhesions. It is worth noting that cases with clinical or pathologic evidence suggestive of secondary FSGS (e.g., reflux nephropathy, renal hypodysplasia, severe obesity, cyanotic heart disease, and unilateral renal agenesis) and also those with congenital forms of the FSGS were not included in this study.

Demographic, clinical, and laboratory data of eligible cases were collected retrospectively by review of medical records. These data comprised height, weight, age at onset, 24-h urinary protein excretion, serum albumin, serum creatinine, creatinine clearance (based on Schwartz formula), and systolic and diastolic blood pressure. It must be noted that these data were updated regularly (every 3-6 months), during follow-up visit for each patient.

Definitions

Chronic kidney disease (CKD) was defined as glomerular filtration rate (GFR) lower than 90 ml/min/1.73m^2. Hypertension was defined as blood pressure higher than 95th percentile for age and height according to data from the Task Force Report on High Blood pressure in Children and Adolesents.[22] Response to treatment was classified as complete remission, partial remission, and non-remission: Complete remission was defined as negative or trace proteinuria adjusted by Body Surface Area <350 mg/m^2/day. Partial remission was defined as a reduction in proteinuria but still remaining in the supranormal range. Non-responder (steroid resistant nephrotic syndrome [SRNS]) was considered as inability to induce remission and/or partial remission within 4 weeks of daily steroid therapy followed by three pulses of methyprednisolon. Steroid sensitive nephrotic syndrome (SSNS) was defined as remission after 4-6 weeks of full dose of steroid therapy.

Treatment protocols

All patients underwent similar initial treatment strategy. They were initially treated with oral 60 mg/m^2/day prednisolone for 4-6 weeks, with or without three consecutive doses of methyl prednisolone pulses (10-30 mg/kg/dose). This was then tapered in an alternate-day regimen over six to nine months.

Patients who responded partially to prednisolone or demonstrated steroid side effects received cyclophosphamide (2-3 mg/kg/day for 2-3 months). For those patients who did not respond to aforementioned medications, cyclosporine A (3-5 mg/kg/day) was commenced after performing kidney biopsy. In cases that did not respond to cyclosporine A in a 6-month course, mycophenolate mofetil (500-1,000 mg/m^2/day) was replaced.

Angiotensin converting enzyme inhibitor (ACEI) was added as an adjuvant therapy to control hypertension or proteinuria.

Tissue specimens and immunohistochemistry

A total of 31 formalin-fixed, paraffin-embedded kidney biopsy sections (3 μm) were used for immunohistochemical staining. The following monoclonal antibodies (mAb) were used: (i) Monoclonal mouse anti-SMA1 antibody (Dako, Clone 1A4, Code M0851) at a running dilution

of 1:50. Studies have generally accepted its specificity for smooth muscle actin.[23] (ii) Monoclonal mouse anti-human vimentin antibody (Dako, Clone V9, code M 0725) at a running dilution of 1:200. This antibody is commonly used to stain glomerular epithelial cells.[24] (iii) Monoclonal mouse anti-human desmin antibody (Dako Clone D 33, code M 0760) at a running dilution of 1:50. It has shown specificity for staining glomerular epithelial cell in rats.[25]

Double immunohistochemistry, using a microwave-based two color staining was applied.[26] Tissue sections were baked, dewaxed, hydrated to distilled water, and then pre-incubated in 10% FCS and 10% normal goat serum to block non-specific binding. After applying primary antibodies (anti α-SMA, desmin and vimentin m-Ab), the samples were incubated for 30 minutes at room temperature. Then the slides were washed two times in Tris Buffer Saline (TBS) and quenched with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase. The sections were incubated for 30 min after applying secondary antibody (goat anti-mouse IgG, DAKO). After rinsing 3 times with TBS, mouse PAP in a dilution of 1/50 was used. These steps were followed using diaminobenzidine (DAB) for 5 min to produce a light brown color. The slides were rinsed in tap water for 5 min. Finally, the slides were counter-stained with hematoxylin (blue) and then dehydrated and mounted with entelan glue.

Quantitation of tissue staining

All of the specimens were reviewed by a pathologist who was blinded to the clinical features and outcome of patients. The immunohistochemical staining was estimated stereologically by a graticule provided 121 points each field that was superimposed upon the specimen.

For each specimen, the following features were recorded:

A. Scoring for tubular staining with mAB in each tubular cross-section:
   - 0 epithelial tubular cell stained =0
   - 1-2 epithelial tubular cells stained = +1
   - >2 epithelial tubular cells and less than 50% of cells stained = +3
   - More than 50% of epithelial tubular cells stained = +4

B. At least 100 tubules and at most 500 tubules were scored and the average was reported as the final score:
   Scoring for interstitial staining with monoclonal antibodies after overlaying an ocular grid:
   - Each field (grid) =121 points
   - A = number of positive points that matched completely with positive cells
   - B = number of non-scored points
   - C = number of assessed points (121-B)
   - D = percentage of positive points = A/C
   - Total score = D1+D2+…+Dn/total number of fields

Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 16). The categorized data were reported as frequencies and percentages. Continuous data were reported as mean and standard deviation (SD). The renal survival was evaluated using the Kaplan-Meier method. To determine the predictive value of each variable for the outcome, the Cox regression was used. Log rank test was used to compare renal survival between steroid responders and non-responders and also among patients reaching complete remission and partial remission. Pearson's correlation test was used to analyze correlations. In addition, we used receiver operator characteristic curve analysis to detect the best cut-off values for cytoskeletal proteins (vimentin and α-SMA) to predict the outcome. A *P* value lower than 0.05 was considered as a significant threshold.

Ethics

Written informed consent from all parents and oral assent from children above six years of age were obtained for this study. This survey was performed in accordance with the Helsinki Declaration.

Results

Twenty out of the 31 patients were males (64.5%). The ages in males and females were 56.3 ± 41.4 and 78.0 ± 60.4 months, respectively. The duration of follow-up was 46.3 ± 56.5 months. During the observation period, three of our patients died. The cause of death in one of them was massive brain emboli and in two others was severe electrolytes imbalance.

Clinical features

In this study, 16 (51.6%) and 6 (19.3%) cases reached complete and partial remission respectively. Cumulative incidence of remission was 22 (70.9%). However, five patients (16.1%) did not reach remission during the observation period. In addition, the majority of our patients were categorized as non-responder to steroid therapy (67.7%).

Twenty six out of 31 patients (83.8%) received angiotensin receptor blockers (ARBs) or ACEI for treatment of hypertension and/or to decrease the amount of proteinuria.

Further laboratory and clinical features of the patients, both in the time of presentation and in our final evaluation, are summarized in Table 1. The results of analysis (Inter model) revealed that response to steroid and the last diastolic blood pressure were predictive variables for the last GFR ($P < 0.05$).

Renal survival

Among the patients, 51% progressed to CKD. The 1-year and 5-year renal survival rates in our study were 86.7% and 41.7%, respectively [Figure 1]. These rates were not significantly different between two sexes ($P > 0.005$). The median renal survival was 60 ± 29.5 months.

The median of renal survival in steroid responders (33.3% of patients) and non-responders (66.6% of patients) were 161 ± 10.2 and 25 ± 3.05 months, respectively [Figure 2].

Histopathology

The mean number of assessed glomeruli (in each specimen) was 16.5 ± 9.6. Interstitial fibrosis and tubular atrophy were reported in 42% and 54% of patients respectively. Almost all patients who had interstitial fibrosis were also reported to have tubular atrophy. However, vessel changes only were reported in three patients (9.6%). In the non-responder patients, Multiple Regression Analysis showed interstitial fibrosis as the only histopathologic predictor of non-response to steroid and diastolic and systolic hypertension ($P < 0.05$).

Immunohistochemistry findings

Light microscopic and immunohistochemical staining for vimentin and α-SMA are shown in Figure 3. In stained specimens, mean vimentin expression score for interstitium and tubules were 0.19 ± 0.17 and 2.24 ± 0.78 respectively.

The mean score of vimentin and α-SMA expression in interstitium were not significantly different between patients who had GFR lower or greater than 90 ml/min. Similar results were obtained considering GFR lower or greater than 70 ml/min and 50 ml/min (the results are not shown).

The mean point counting scores for α-SMA were 6.17 ± 5.97 and 0.27 ± 0.13 for glomeruli and interstitial respectively. Desmin was not detected in any of the

Table 1: Clinical characteristics of the patients participated at the study

Characteristics	At presentation	At last visit	*P* value
Height (cm)	1.13±23.87	1.24±24.39	-
Weight (kg)	23.22±12.37	30.59±14.0	-
Systolic BP (mm Hg)	109.58±12.33	106.48±23.85	NS
Diastolic BP (mm Hg)	71.23±9.60	77.13±8.71	S
Creatinin (mg/dL)	0.69±0.15	2.16±6.19s	
Albumin (g/dL)	2.14±0.94	3.52±1.19	NS
BUN (mg/dL)	21.06±9.96	23.93±13.07	S
Pr.BSA (g/day)	2.29±1.04	0.66±0.91	S
GFR (ml/min per 1.73 m^2 BSA)	97.53±23.64	88.36±35.24	S
BMI	17.42±4.82	17.40±3.45	-

Data are reported as Mean±standard deviation, BP: Blood pressure, NS: Non-significant, S: Significant, BUN: Blood urea nitrogen, Pr.BSA: Proteinuria adjusted by body surface area

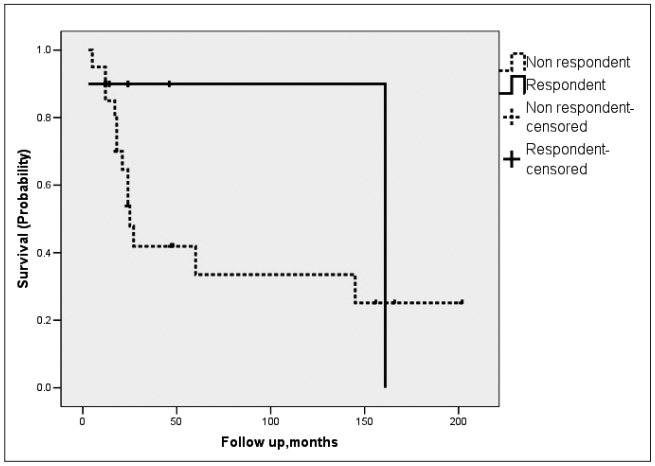

Figure 1: Median Renal Survival in children with focal segmental glomerulosclerosi

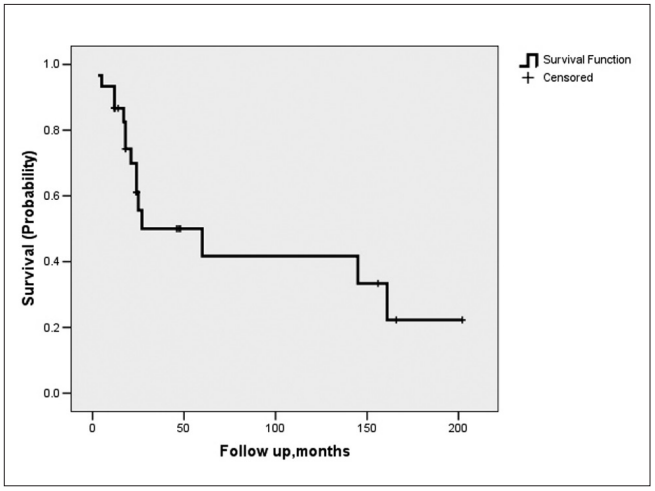

Figure 2: Renal survival according to response to steroid

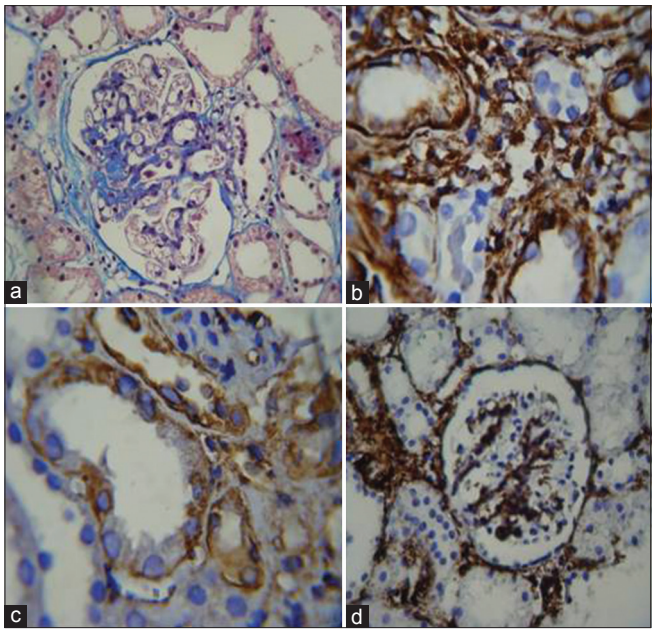

Figure 3: (a) Focal segmental glomerulosclerosis (FSGS), light microscopic examination showing collapse of capillaries together with mesangial matrix expansion (masson trichrome staining, original magnification, ×400). (b) Staining for vimentin, using an anti-vimentin antibody, showing an increase in interstitium of a case with FSGS (original magnification, ×400). (c) Immunostaining for vimentin showing positivity in tubular epithelial cells of a patient with FSGS (original magnification, ×400). (d) Positive α SMA immunostaining in mesangial and epithelial cells of a FSGS glomeruli (original magnification, ×400)

tubular, interstitial, or glomerular staining. Tubular vimentin expression was correlated with systolic blood pressure at presentation ($P = 0.01$, $r = 0.46$). Interstitial vimentin expression was correlated with systolic blood pressure at presentation and also systolic and diastolic blood pressure at last visit ($r^1 = 0.37$, $r^2 = 0.4$, $r^3 = 0.5$ respectively). Interstitial α-SMA expression was only correlated with the last evaluated diastolic blood pressure ($P = 0.015$, $r = 0.46$). α-SMA interstitial staining was correlated with vimentin interstitial staining ($P = 0.010$, $r = 0.478$). The last evaluated GFR was only correlated with tubular expression of vimentin, $P < 0.05$. These findings were approved by regression analysis, as well.

However, multiple regression analysis did not show any significant prognostic value for the aforementioned pathologic factors.

Discussion

In this study, we evaluated the prognostic values of certain clinical and laboratory features and myofibroblastic cytoskeletal filaments (α-SMA, desmin, and vimentin) expression in kidney tissues of FSGS children for renal survival. FSGS is the most prevalent glomerular disease leading to ESRD in children. In the recent decades the incidence of this disease has increased.[19] Following items protend a poorer prognosis: The persistence of the nephrotic range of proteinuria, no response to steroid, non-remittent disease, American-African race, serum creatinine more than 1.5 mg/dl at presentation, and the higher extent of interstitial fibrosis.[12,27,28] However, there is no report regarding the assessment of the role of cytoskeletal proteins in predicting the outcome.

In our study, the last evaluated GFR was correlated with the following clinical and histopathologic findings: diastolic blood pressure, response to steroids, extent of interstitial fibrosis, and presence of tubular atrophy. Those patients, who did not respond to steroids, had higher diastolic blood pressures and lower GFRs in the last evaluations. Comparing with our previous report, the patients in this study reached a higher rate of complete remission.[15]

This can be due to prescription of new medications such as rituximab and MMF, early administration of ACEIs, and addition of a combination of ARBS to conventional treatment protocols such as cyclosporine.

Shiiki *et al.*[29] reported a poorer outcome among patients with first serum creatinine more than 1.5 gm/dl, patients with interstitial fibrosis in histopathologic findings, and in treatment-resistant patients. Various studies demonstrated poor renal survival associated with low GFR at the onset of disease.[6,8,30]

However, in agreement with the results of Banfi *et al.*,[31] our findings did not support the role of GFR at onset in predicting the disease outcomes.

It must be noted that administration of ARBs or ACEIs in patients with low effective blood volume and even mild degree of ATN may explain the temporary and reversible low GFR at the time of presentation.

There is consensus among most studies regarding the role of response to steroid in predicting the renal survival.[2,6,8,30,31] We achieved similar result by multivariate analysis [Figure 2].

In glomerular diseases, tubulointerstitial damages are provoked through the following mechanisms: Impaired glomerular permselectivity, altered glomerular hemodynamics, immunologic mechanism, inflammatory mediators, and nephron loss.[32] It is demonstrated that interstitial fibrosis accompanying with tubulointerstitial involvement is an independent predictor of renal impairment.[32] In our study similar to many previous studies, the independent role of interstitial fibrosis in promoting renal impairment was demonstrated.

Vimentin is a sub-unit protein of the intermediate filaments. One of its functions in mesenchymal cells is intracellular transport of proteins between the nucleus and plasma membrane. In addition, in both infants' and adults' kidney tissues, it has been shown that vimentin is present in podocytes and glomeruli, but not in interstitium.[16,18,33] However, vimentin was not reported to have a major role in functioning of foot processes.[34] Tubular neo-expression of vimentin has been shown in diabetic nephropathy and some other immune-mediated and non-immune-mediated tubular injuries. The expression of vimentin in the injured tubules may coincide with transdifferentiation of dedifferentiated tubular cells into fibroblasts.[35,36] Ostalska-Nowicka *et al.,*[37] reported that podocytes vimentin expression did not differ between mature and immature forms of mesangial glomerulonephritis. Although, we demonstrated vimentin expression both in tubules and interstitium of kidney specimens, its prognostic value in determining renal survival was not shown to be significant.

Desmin, a cytoskeletal protein of the class III intermediate filament is found in muscle cells. In adult, striated muscles form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z line structures. Desmin expression was negative in fetal, adult, and glomerulonephritis kidney samples.[16] Its expression was reported to be associated with early electron microscopic alterations of the podocytes and heavy proteinuria in patients with idiopathic membranous nephropathy.[38] No desmin m-Ab staining was shown in either glomeruli or tubules in our study. However, it is believed that desmin immunostaining can be more detectable is frozen section samples.[39]

According to several lines of evidence, interstitial staining is positive in fibrotic areas of the diseased kidneys, and is associated with increased number of interstitial myofibroblasts.[16,20] The interaction between cytokines and renal cells (fibroblasts, mesangial, and tubular cells) may increase collagen and cellular matrix production by inducing α-SMA expression.[40,41] Positive glomerular staining with α-SMA has been reported in FSGS and proliferative glomerulonephritis.[16,21] Zhang *et al.,*[41] detected α-SMA expression in the interstitium and glomeruli of rats with experimental FSGS. In addition, they suggested that glomerular α-SMA immunostaining is a predictor for renal impairment and glomerulosclerosis. In Hewitson and Becker study, interstitial α-SMA staining was greater in IgA nephropathy when comparing with the control group.[42] Boukhalfa *et al.,*[43] demonstrated that glomerular expression of α-SMA was correlated with activated glomerular mesangial cells, but not with glomerulosclerosis. However, up-regulation of interstitial α-SMA had correlation with the degree of interstitial fibrosis. Furthermore, glomerular α-SMA expression has been shown in phenotypically changed mesangial cells to myofibroblasts in primary FSGS.[44]

Geleilete *et al.,*[21] did not find any correlation between the expression of glomerular and interstitial expression of α-SMA and glomerulosclerosis and/or interstitial fibrosis. In addition, α-SMA expression was not correlated with blood pressure at the time of biopsy and response to treatment.

In our study, α-SMA staining was demonstrated in both glomeruli and interstitial. Although interstitial α-SMA staining as a marker of interstitial fibrosis was correlated with the last mean blood pressure, ANOVA regression analysis did not show its significance as an independent factor.

Conclusion

No cytoskeletal protein expression as an independent predictor for renal survival was demonstrated. Further studies with larger sample size and longer follow-up periods are warranted to investigate the prognostic values of other histopathologic features in pediatrics with FSGS.

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