EKSPRESIJA NEURALNOG ĆELIJSKOG ADHEZIONOG MOLEKULA U INTERSTICIJUMU BUBREGA SA RAZLIČITIM STEPENOM FIBROZE

ORIGINALNI RAD

ORIGINAL ARTICLE

EXPRESSION OF THE NEURAL CELL ADHESION MOLECULE IN THE RENAL INTERSTITIUM IN DIFFERENT STAGES OF FIBROSIS

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SAŽETAK

Uvod: U zdravim adultnim bubrezima diferentovane tubulske epitelne ćelije ne eksprimiraju neuralni ćelijski adhezioni molekul (engl. *neural cell adhesion molecule – NCAM*), dok se malobrojne *NCAM* eksprimirajuće ćelije mogu detektovati u bubrežnom intersticijumu. Uloga i značaj ovih ćelija još nisu razjašnjeni, ali je primećeno da se broj *NCAM* eksprimirajućih ćelija povećava u stadijumu početne intersticijske fibroze.

Cilj rada: Cilj rada je da se ispita značaj ekspresije *NCAM* molekula u intersticijumu bubrega kod etiološki različitih oboljenja sa različitim stepenom intersticijske fibroze i da se definišu patohistološki i klinički pokazatelji (prediktori) oštećenja funkcije bubrega.

Materijal i metode: Ispitivanje je obuhvatilo 69 pacijenata kojima su iglene biopsije bubrega urađene tokom 2011. i 2012. godine. Prikupljeni su klinički i laboratorijski podaci u vreme biopsije i u vreme poslednjeg kontrolnog pregleda. Patohistološke karakteristike definisane su optičko-mikroskopski, dok su imunohistohemijskim bojenjem, korišćenjem primarnog *NCAM* antitela (1:50, klon *123C3.D5*), detektovane *NCAM* eksprimirajuće intersticijske ćelije.

Rezultati: *NCAM* eksprimirajuće intersticijske ćelije detektovane su u 59,4% biopsija bubrega, a prisustvo ovih ćelija bilo je značajno češće u početnim fazama intersticijske fibroze nego u drugim stadijumima ($p < 0,001$) i nije zavisilo od patohistološke dijagnoze ($p = 0,995$). Pacijenti kod kojih su detektovane *NCAM* ćelije imali su znatno niže vrednosti proteinurije u vreme biopsije u odnosu na pacijente bez *NCAM* intersticijskih ćelija ($p = 0,024$). Vrednosti serumskog kreatinina ($p < 0,001$) i uree ($p = 0,007$) značajno su uticale na verovatnoću pogoršanja bubrežne funkcije.

Zaključak: Prisustvo *NCAM* ćelija u u intersticijumu bubrega je karakteristika ranih faza hroničnih bolesti bubrega sa početnom intersticijskom fibrozom i blažim stepenom proteinurije.

Ključne reči: *NCAM* eksprimirajuće ćelije, intersticijska fibroza, bubrežno oštećenje, prediktori

ABSTRACT

Introduction: In healthy adult kidneys, differentiated tubular epithelial cells do not express the neural cell adhesion molecule (NCAM), while a small number of NCAM-expressing cells can be detected in the renal interstitium. The role and the significance of these cells have not yet been clarified, but it has been observed that the number of NCAM-expressing cells increases in the initial stage of interstitial fibrosis.

Aim: The aim of the study is to examine the significance of the expression of NCAM molecules in the renal interstitium, in etiologically different diseases, with varying degrees of interstitial fibrosis, as well as to define the pathohistological and clinical indicators (predictors) of impaired kidney function.

Materials and methods: The study included 69 patients who underwent needle biopsies of the kidneys in 2011 and 2012. Clinical and laboratory data were collected at the time of the biopsy and at the time of the latest follow-up examination. Pathohistological characteristics were defined optically-microscopically, while NCAM-expressing interstitial cells were detected with immunohistochemical staining, using the primary NCAM antibody (1:50, clone 123C3.D5).

Results: NCAM-expressing interstitial cells were detected in 59.4% of kidney biopsies, the presence of these cells was significantly more frequent in the initial stages of interstitial fibrosis than in the remaining stages ($p < 0.001$), and it did not depend on the pathohistological diagnosis ($p = 0.995$). Patients in whom NCAM cells were detected had significantly lower proteinuria levels at the time of biopsy, as compared to patients without NCAM interstitial cells ($p = 0.024$). The levels of serum creatinine ($p < 0.001$) and urea ($p = 0.007$) significantly influenced the probability of the deterioration of renal function.

Conclusion: The presence of NCAM cells in the kidney interstitium is a characteristic of the early stages of chronic kidney disease with incipient interstitial fibrosis and a lesser degree of proteinuria.

Keywords: NCAM-expressing renal interstitial cells, interstitial fibrosis, renal impairment, predictors

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UVOD

Neuralni ćelijski adhezioni molekul (engl. *neural cell adhesion molecule – NCAM*) je sijaloglikoprotein uključen u ćelijsko-ćelijske interakcije i u interakcije ćelije sa ekstracelularnim matriksom [1,2].

Ovaj molekul ima veoma značajnu ulogu u toku embrionalnog razvoja mozga, perifernih nerava, mišića i bubrega [3]. Ćelije metanefričnog mezenhima obilno eksprimiraju *NCAM*, ali se ekspresija ovog molekula postepeno gubi sa napredovanjem diferencijacije ćelija koje će u najvećoj meri graditi epitel tubula bubrega [4]. U zdravim adultnim bubrezima, diferentovane epitelne ćelije ne eksprimiraju *NCAM* [5]. Međutim, malobrojne *NCAM* eksprimirajuće ćelije se mogu detektovati u intersticijumu zdravih bubrega, i za njih se smatra da predstavljaju ćelije metanefričnog mezenhima koje su zaostale nakon embrionalnog razvoja bubrega [3,4]. Uloga i značaj ovih ćelija nisu razjašnjeni ali je primećeno da se broj *NCAM* eksprimirajućih ćelija povećava u stadijumu početne intersticijske fibroze, te se sve više spekuliše o značaju ovih ćelija u ranoj fazi reparacije bubrežnog parenhima [4]. Ovoj hipotezi doprinose i rezultati drugih istraživača koji su ukazali na značaj *NCAM* molekula u procesu fibroze skeletnih mišića i pankreasa [6].

NCAM je takođe i specifični marker neuroektodermalne i neuroendokrine diferencijacije ćelija [1,2]. S obzirom na to da se pretpostavlja da eritropoetin produkujuće ćelije u bubrezima vode poreklo od neuralnog grebena, tj. da imaju neuroektodermalno poreklo [7,8,9], nije isključena mogućnost da *NCAM* ekprimirajuće intersticijske ćelije zapravo predstavljaju eritropoetin produkujuće ćelije. Zapažena je indukcija transkripcije nekoliko *NCAM* izoformi zajedno sa *FGFR1* receptorom, što ukazuje na mehaničku vezu između *NCAM/FGFR1* signalizacije i indukcije fibrogeneze u bubrezima [10].

Imajući u vidu da je intersticijska fibroza patomorfološki supstrat narušavanja funkcije bubrega i da se javlja kod mnogih, etiološki raznovrsnih oboljenja bubrega, ulažu se veliki napori u ispitivanje molekurne osnove i signalnih puteva koji doprinise ovom procesu. Stoga je cilj ove studije bio da se ispita značaj ekspresije *NCAM* molekula u intersticijumu bubrega kod etiološki raznovrsnih oboljenja bubrega sa različitim stepenom intersticijske fibroze i da se definišu kliničke karakterstike bolesnika kod kojih su detektovane ove ćelije. Takođe, ova studija je sprovedena i sa ciljem da se definišu pokazatelji (prediktori) narušavanja funkcije bubrega.

MATERIJAL I METODE

Optičko-mikroskopski su retrospektivno analizirane iglene biopsije bubrega pacijenata dijagnostikovanih tokom 2011. i 2012. godine, na Institutu za patologiju Medicinskog fakulteta, Univerziteta u Beogradu. Odabrano je 69

INTRODUCTION

Neural cell adhesion molecule (NCAM) is a sialoglycoprotein involved in cell-cell interactions and cell interactions with the extracellular matrix [1,2].

This molecule plays a very important role in the embryonic development of the brain, the peripheral nerves, the muscles and the kidneys [3]. The cells of the metanephric mesenchyme abundantly express NCAM, but the expression of this molecule gradually disappears with the progression of the differentiation of the cells that will predominantly build the renal tubular epithelium [4]. In healthy adult kidneys, differentiated epithelial cells do not express NCAM [5]. However, a small number of NCAM-expressing cells can be detected in the interstitium of healthy kidneys, and they are thought to represent metanephric mesenchymal cells left behind after embryonic kidney development [3,4]. The role and the importance of these cells have not as yet been clarified, but it has been observed that the number of NCAM-expressing cells increases in the initial stage of interstitial fibrosis, and there is increasing speculation about the importance of these cells in the early stage of renal parenchymal repair [4]. This hypothesis is supported by the results of other researchers who have indicated the importance of the NCAM molecule in the process of skeletal muscle and pancreatic fibrosis [6].

NCAM is also a specific marker of neuroectodermal and neuroendocrine cell differentiation [1,2]. Given that it is assumed that the erythropoietin-producing cells in the kidneys originate from the neural crest, i.e., they are believed to have neuroectodermal origin [7,8,9], the possibility that NCAM-expressing interstitial cells actually represent erythropoietin-producing cells should not be excluded. Transcriptional induction of several NCAM isoforms was observed along with FGFR1, suggesting a mechanical link between NCAM/FGFR1 signaling and fibrogenesis induction in the kidney [10].

Bearing in mind that interstitial fibrosis is a pathomorphological substrate of impaired kidney function and that it occurs in many, etiologically diverse kidney diseases, significant effort is being made to investigate the molecular basis and the signaling pathways contributing to this process. Therefore, the aim of this study was to examine the significance of the expression of NCAM molecules in the kidney interstitium in etiologically diverse kidney diseases with different degrees of interstitial fibrosis, as well as to define the clinical characteristics of patients in whom these cells were detected. Also, this study was conducted with the aim of defining indicators (predictors) of impaired kidney function.

pacijenata kod kojih je bilo dovoljno tkiva u parafinskim kalupima biopsija iz kojih bi se izradile pločice za imunohistohemijsko bojenje. Takođe, pregledom istorija bolesti ovih 69 pacijenata, prikupljeni su relevantni klinički i laboratorijski podaci zabeleženi u vreme biopsije, kao i podaci sa poslednje redovne kontrole pacijenata.

Iz parafinskih blokova su izrađene pločice sa tkivom sečenim na debljinu od 5 μm. Nakon deparafinizacije u ksilolu i hidratacije, pločice su ubačene u citratni pufer (pH = 6,0) i izložene mikrotalasima u trajanju od 20 minuta na 400 W. Blokada peroksidazne aktivnosti je izvršena sa 1% *BSA* (engl. *bovine serum albumin – BSA*). Nakon demaskiranja antigena urađena je inkubacija sa primarnim *NCAM* antitelom (1:50, klon 123C3.D5, *LabVision, USA*) u trajanju od 60 minuta. *EnVisionTM* (*DAKO*, Danska) je korišćen za vizualizaciju antigen-antitelo reakcije sa 3,3'-diaminobenzidinom (DAB), nakon čega je usledilo kontrastiranje hematoksilinom. Negativne kontrole dobijene su izostavljanjem primarnog antitela, a kao pozitivna kontrola korišćeno je tkivo fetalnog bubrega. Pločice su pregledane upotrebom *BX53* svetlosnog mikroskopa sa *DP12CCD* kamerom (*Olympus*, Nemačka). Broj *NCAM* eksprimirajućih ćelija je izražen kao broj ćelija po vidnom polju pri uveličanju ×400.

Stepen intersticijske fibroze (engl. *interstitial renal fibrosis – IRF*) je određivan semikvantitativno, primenom skora od 0 do 3, pri čemu: 0 – nema intersticijske fibroze; 1 – manje od 25% tkiva zahvaćeno intersticijskom fibrozom; 2 – između 25% i 50% tkiva zahvaćeno intersticijskom fibrozom; 3 – više od 50% tkiva zahvaćeno intersticijskom fibrozom. Za klasifikovanje pacijenata u stadijume hronične bolesti bubrega (engl. *chronic kidney disease – CKD*), korišćen je najpoznatiji svetski vodič [11].

Statistička analiza je urađena upotrebom *IBM SPSS* softvera, verzije 20.0. U zavisnosti od prirode posmatrane varijable i broja ispitivanih grupa, za ispitivanje razlike među grupama, korišćeni su $\chi 2$ test, Studentov t test i Men-Vitnijev U test. Za određivanje prediktora pogoršanja funkcije bubrega, korišćena je Kaplan-Majerova univarijantna analiza preživljavanja. Statistički značajnim smatrano je $p < 0,05$.

REZULTATI

NCAM eksprimirajuće intersticijske ćelije detektovane su u 59,4% biopsija bubrega, a prisustvo ovih ćelija nije zavisilo od patohistološke dijagnoze ($p = 0,995$). Međutim, utvrđeno je da je učestalost pozitivnih biopsijskih slučajeva bila statistički značajno veća kod pacijenata koji su imali početnu intersticijsku fibrozu (*IRF-1*) u odnosu na pacijente u drugim kategorijama *IRF* ($p < 0,001$; Tabela 1). Na Slici 1 prikazane su *NCAM* eksprimirajuće intersticijske ćelije u biopsijskim uzorcima sa intersticijskom fibrozom.

MATERIALS AND METHODS

Needle biopsies of kidneys of patients diagnosed during 2011 and 2012 at the Institute of Pathology of the Faculty of Medicine, University of Belgrade, were retrospectively analyzed optically and microscopically. Sixty-nine patients, who had enough tissue in paraffin-embedded biopsies to make slides for immunohistochemical staining, were selected. Also, by reviewing the medical histories of these 69 patients, relevant clinical and laboratory data recorded at the time of the biopsy, as well as data from the latest regular follow-up of the patients, were collected.

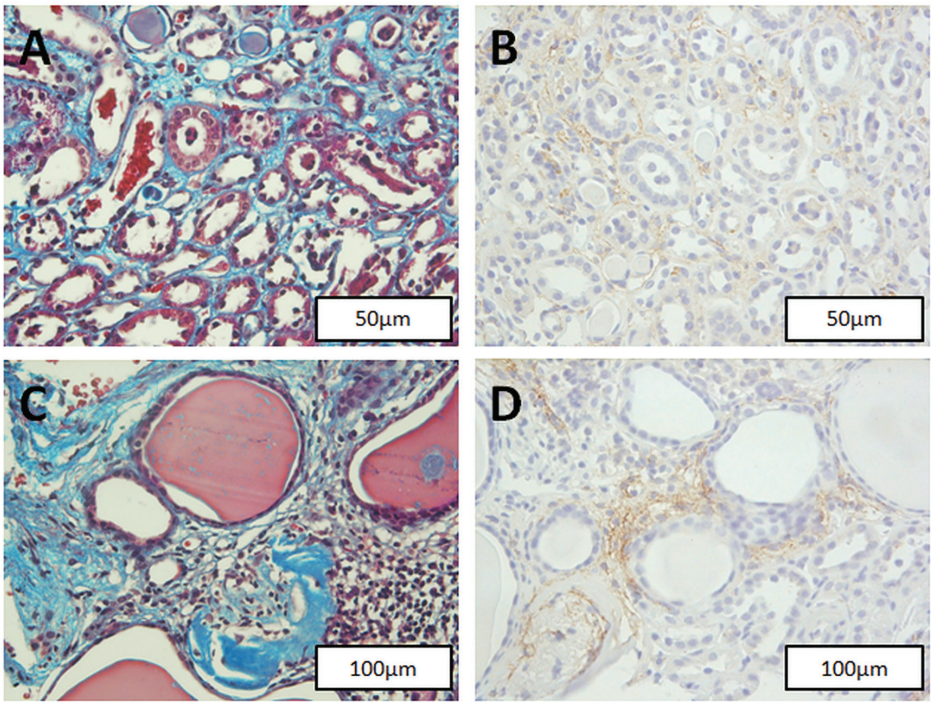
Plates with tissue cut to a thickness of 5 μm were made from paraffin blocks. After deparaffinization in xylene and hydration, the slides were placed in a citrate buffer (pH = 6.0) and exposed to microwaves for 20 minutes at 400 W. Peroxidase activity was blocked with 1% bovine serum albumin (BSA). After antigen unmasking, incubation with the primary NCAM antibody (1:50, clone 123C3.D5, LabVision, USA) was performed for 60 minutes. EnVisionTM (DAKO, Denmark) was used to visualize the antigen-antibody reaction with 3,3'-diaminobenzidine (DAB), upon which counterstaining with hematoxylin was performed. Negative controls were obtained by omitting the primary antibody, and fetal kidney tissue was used as a positive control. Plates were examined using a BX53 light microscope with a DP12CCD camera (Olympus, Germany). The number of NCAM-expressing cells was presented as the number of cells per field of view at ×400 magnification.

The degree of interstitial renal fibrosis (IRF) was determined semiquantitatively, using a score from 0 to 3, whereby the following applied: 0 – no interstitial fibrosis; 1 – less than 25% of tissue affected by interstitial fibrosis; 2 – between 25% and 50% of tissue affected by interstitial fibrosis; 3 – more than 50% of tissue affected by interstitial fibrosis. The best-known international guide was used to classify patients according to the stages of chronic kidney disease (CKD) [11].

Statistical analysis was performed using the IBM SPSS software, version 20.0. Depending on the nature of the observed variable and the number of investigated groups, the $\chi 2$ test, Student's t test and Mann-Whitney U test were used to examine the difference between the groups. Kaplan-Meier univariate survival analysis was used to determine predictors of the deterioration of renal function, whereby $p < 0.05$ was considered statistically significant.

RESULTS

NCAM-expressing interstitial cells were detected in 59.4% of kidney biopsies, and the presence of these cells did not depend on pathohistological diagnosis



Slika 1. Prisustvo NCAM eksprimirajućih intersticijskih ćelija u biopsijskim uzorcima sa fibrozom intersticijuma: (A) *Masone-trichrome* bojenje, ×200; (B) imunohistohemijsko bojenje *NCAM* antitelom (1:50, klon 123C3.D5), ×200; (C) *Masone-trichrome* bojenje, ×400; (D) imunohistohemijsko bojenje *NCAM* antitelom (1:50, klon 123C3.D5), ×400

Figure 1. Presence of NCAM-expressing interstitial cells in biopsy specimens with interstitial fibrosis: (A) Masson's trichrome staining, ×200; (B) immunohistochemical staining with the NCAM antibody (1:50, clone 123C3.D5), ×200; (C) Masson's trichrome staining, ×400; (D) immunohistochemical staining with the NCAM antibody (1:50, clone 123C3.D5), ×400

Tabela 1. Distribucija pacijenata sa NCAM eksprimirajućim intersticijskim ćelijama u odnosu na patohistološke parametre

Table 1. Distribution of patients with NCAM-expressing interstitial cells in relation to pathohistological parameters

Patohistološke parametri / *Pathohistological parameters*		**NCAM eksprimirajuće intersticijske ćelije / *NCAM-expressing interstitial cells* n (%)**		**p-vrednost/ *p-value***
		Odsutna / *Absent*	**Prisutna / *Present***	
Dijagnoza / *Diagnosis*	Fokalno-segmentna glomeruloskleroza / *Focal segmental glomerulosclerosis*	5 (38.8%)	8 (61.5%)	0.995
	Transplantirani bubreg / *Transplanted kidney*	5 (71.4%)	2 (28.6%)	
	Membranozni GN / *Membranous GN*	4 (33.3%)	8 (66.7%)	
	Lupusni nefritis / *Lupus nephritis*	5 (31.2%)	11 (68.8%)	
	Mezangioproliferativni GN / *Mesangioproliferative GN*	0 (0.0%)	4 (100.0%)	
	Membranoproliferativni GN / *Membranoproliferative GN*	2 (33.3%)	4 (66.7%)	
	Minimalne promene / *Minimal changes*	3 (100.0%)	0 (0.0%)	
	IgA nefropatija / IgA nephropathy	2 (66.7%)	1 (33.3%)	
	Rapidno-progresivni GN / Rapidly progressive GN	2 (40.0%)	3 (60.0%)	
Stadijum intersticijske fibroze / *Stage of interstitial fibrosis*	*IRF – 0*	14 (70.0%)	6 (30.0%)	0.001*
	IRF – 1	4 (13.8%)	25 (86.2%)	
	IRF – 2	4 (44.4%)	5 (55.6%)	
	IRF – 3	6 (54.5%)	5 (45.5%)	

*Grupe za koje je razlika bila statistički značajna; IRF – intersticijska fibroza bubrega (engl. *interstitial renal fibrosis*)

*Groups for which the difference was statistically significant; IRF – interstitial renal fibrosis

Tabela 2. Distribucija pacijenata sa NCAM eksprimirajućim intersticijskim ćelijama u odnosu na kliničke i laboratorijske parametre u vreme biopsije

Table 2. Distribution of patients with NCAM-expressing interstitial cells in relation to clinical and laboratory parameters at the time of biopsy

Klinički i laboratorijski parametri u vreme biopsije / *Clinical and laboratory parameters at the time of biopsy*		NCAM eksprimirajuće intersticijske ćelije / *NCAM-expressing interstitial cells*		p-vrednost/ *p-value*
		Odsutna / *Absent*	Prisutna / *Present*	
CKD stadijum n (%) / *CKD stage n (%)*	*CKD 1*	12 (42.9%)	16 (57.1%)	0.954
	CKD 2	3 (33.3%)	6 (66.7%)	
	CKD 3	3 (50.0%)	3 (50.0%)	
	CKD 4	5 (55.6%)	4 (44.4%)	
	CKD 5	2 (28.6%)	5 (71.4%)	
Serumski kreatinin [µmol/l] / *Serum creatinine level [µmol/l]*		146.64 ± 136.28	200.07 ± 239.45	0.244
Klirens kreatinina [ml/min] / *Creatinine clearance level [ml/min]*		77.56 ± 39.49	104.32 ± 70.19	0.199
eGFR [ml/min/1,73m^2] / *eGFR level [ml/min/1.73m2]*		73.80 ± 47.49	74.56 ± 47.21	0.952
Urea [mmol/l] / *Urea level [mmol/l]*		10.61 ± 8.66	11.037 ± 8.17	0.838
Glukoza [mmol/l] / *Glucose level [mmol/l]*		5.15 ± 1.11	4.76 ± 0.81	0.115
Eritrociturija n (%) / *Erythrocyturia n (%)*	Odsutna / *Absen*	15 (44.1%)	19 (55.9%)	0.585
	Prisutna / *Present*	12 (37.5%)	20 (62.5%)	
Proteinurija [g/24 h] / *Proteinuria [g/24 h]*		8.41 ± 9.45	3.97 ± 2.63	0.024*
Eritrociti [×10^{12}/l] / *Erythrocyte count [×10^{12}/l]*		4.09 ± 0.66	4.09 ± 0.76	0.999
Hemoglobin [g/l] / *Hemoglobin level [g/l]*		122.85 ± 16.77	121.61 ± 25.01	0.852
Hematokrit / *Hematocrit level*		0.35 ± 0.91	0.37 ± 0.07	0.297
MCV [fl] / *MCV [fl]*		88.64 ± 52.45	90.56 ± 4.31	0.231

*Grupe za koje je razlika bila statistički značajna; Proteinurija [g/24 h

*Groups for which the difference was statistically significant; Proteinuria [g/24 h]

Distribucija pacijenata sa *NCAM* eksprimirajućim intersticijskim ćelijama u odnosu na *CKD* stadijum se nije značajno razlikovala, kako u vreme biopsije (p = 0,954; Tabela 2), tako i u vreme poslednje kontrole (p = 0,601; Prilog IV, Tabela 3).

Međutim, uočeno je da su prosečne vrednosti serumskog kreatinina (*sCr*) i klirensa kreatinina (*CCr*), u grupi pacijenata kod kojih su *NCAM* ekprimirajuće ćelije detektovane u intersticijumu (*sCr*: 200,1 ± 239,4; *CCr*: 104,3 ± 70,2) bile veće u odnosu na grupu pacijenata bez *NCAM* eksprimirajućih ćelija u intersticijumu (*sCr*: 146,6 ± 136,3; *CCr*: 77,6 ± 39,5). Sličan trend zabeležen je i na poslednjem kontrolnom pregledu, međutim, razlike nisu bile statistički značajne. Takođe, prosečne vrednosti *eGFR*, uree i glukoze, kao i učestalost javljanja eritrociturije, nisu se statistički značajno razlikovale među poređenim grupama (Tabela 2; Tabela 3).

Pacijenti kod kojih su detektovane *NCAM* eksprimirajuće intersticijske ćelije su imali statistički značajno niže vrednosti proteinurije u vreme biopsije, u odnosu na pacijente u čijim biopsijskim uzorcima nije

(p = 0.995). However, the frequency of positive biopsy cases was found to be statistically significantly higher in patients who had incipient interstitial fibrosis (IRF-1), as compared to patients in other IRF categories (p < 0.001; Table 1). Figure 1 shows NCAM-expressing interstitial cells in biopsy samples with interstitial fibrosis. The distribution of patients with NCAM-expressing interstitial cells in relation to CKD stage did not differ significantly, both at the time of biopsy (p = 0.954; Table 2) and at the time of the latest follow-up (p = 0.601; Table 3).

However, it was observed that the average levels of serum creatinine (sCr) and creatinine clearance (CCr), in the group of patients in whom NCAM-expressing cells were detected in the interstitium (sCr: 200.1 ± 239.4; CCr: 104.3 ± 70.2) were higher, as compared to the group of patients without NCAM-expressing cells in the interstitium (sCr: 146.6 ± 136.3; CCr: 77.6 ± 39.5). A similar trend was observed at the latest follow-up; however, the differences were not statistically significant. Also, the average levels of eGFR, urea and glu-

Tabela 3. Distribucija pacijenata sa NCAM eksprimirajućim intersticijskim ćelijama u odnosu na kliničke i laboratorijske parametre na poslednjem kontrolnom pregledu

Table 3. Distribution of patients with NCAM-expressing interstitial cells in relation to clinical and laboratory parameters at the last follow-up examination

Klinički i laboratorijski parametri na poslednjem kontrolnom pregledu / *Clinical and laboratory parameters at the last follow-up examination*		**NCAM eksprimirajuće intersticijske ćelije / *NCAM-expressing interstitial cells***		**p-vrednost/ *p-value***
		Odsutna / *Absent*	**Prisutna / *Present***	
CKD stadijum n (%) / *CKD stage n (%)*	*CKD 1*	9 (45.0%)	11 (55.0%)	0.601
	CKD 2	1 (14.3%)	6 (85.7%)	
	CKD 3	1 (25.0%)	3 (75.0%)	
	CKD 4	4 (100.0%)	0 (0.0%)	
	CKD 5	2 (22.2%)	7 (77.8%)	
Serumski kreatinin [µmol/l] / *Serum creatinine level [µmol/l]*		183.95 ± 242.20	232.33 ± 317.71	0.560
Klirens kreatinina [ml/min] / *Creatinine clearance level [ml/min]*		86.75 ± 46.34	116.34 ± 74.98	0.334
eGFR [ml/min/1,73m^2] / *eGFR level [ml/min/1.73m2]*		74.59 ± 51.60	69.78 ± 44.31	0.744
Urea [mmol/l] / *Urea level [mmol/l]*		12.24 ± 11.64	12.00 ± 10.50	0.938
Glukoza [mmol/l] / *Glucose level [mmol/l]*		5.27 ± 2.24	4.51 ± 0.57	0.175
Eritrociturija n (%) / *Erythrocyturia n (%)*	**Odsutna / *Absen***	10 (34.5%)	19 (65.5%)	0.547
	Prisutna / *Present*	9 (42.9%)	12 (57.1%)	
Proteinurija [g/24 h] / *Proteinuria [g/24 h]*		2.58 ± 2.36	2.57 ± 2.81	0.993
Eritrociti [×10^{12}/l] / *Erythrocyte count [×10^{12}/l]*		3.43 ± 1.43	4.14 ± 0.78	0.962
Hemoglobin [g/l] / *Hemoglobin level [g/l]*		120.20 ± 17.1	123.71 ± 18.54	0.630
Hematokrit / *Hematocrit level*		0.34 ± 0.05	0.39 ± 0.10	0.211
MCV [fl] / *MCV [fl]*		93.52 ± 4.30	82.97 ± 29.32	0.248

*Grupe za koje je razlika bila statistički značajna; Proteinurija [g/24 h

*Groups for which the difference was statistically significant; Proteinuria [g/24 h]

bilo *NCAM* pozitivnih ćelija u intersticijumu (3,97 g/24 h naspram 8,41 g/24 h; $p = 0,024$), (Tabela 2).

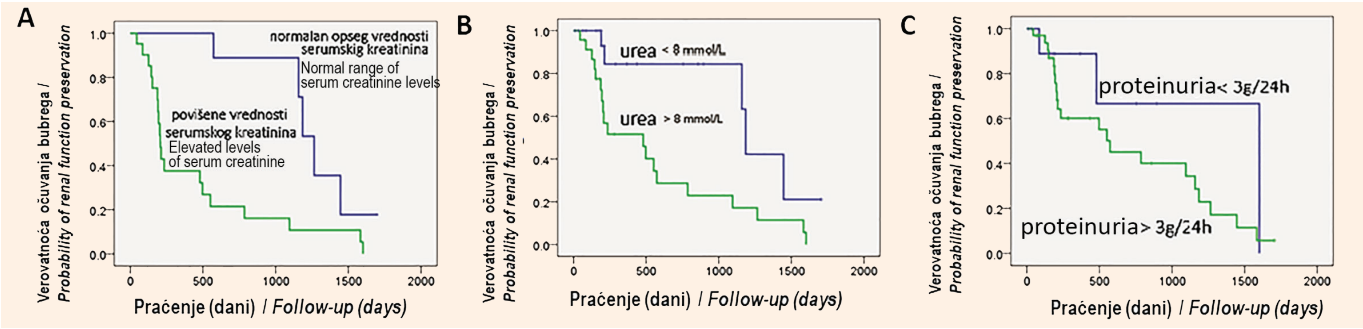
Broj eritrocita, vrednosti hemoglobina, hematokrita i srednje zapremine eritrocita (engl. *mean corpuscular volume – MCV*) se nisu značajno razlikovale među poređenim grupama (Tabela 2; Tabela 3). Pacijenti su prosečno praćeni 16 meseci. Patohistološke i kliničke karakteristike posmatrane su kao potencijalni prediktori progresije *CKD*-a u uznapredovale stadijume. Uočili smo da nijedna osoba sa minimalnim promenama nije progredirala u uznapredovale *CKD* stadijume tokom perioda praćenja, dok je kod svih pacijenata sa rapidno-progresivnim glomerulonefritisom (GN) zabeleženo brzo pogoršanje bubrežne funkcije, međutim, zbog velikog broja patohistoloških dijagnoza i relativno malog broja pacijenata u ovim grupama, statistička analiza nije urađena.

Takođe je primećeno da su pacijenti bez intersticijske fibroze, tokom tri godine praćenja, u 90% slučajeva očuvali bubrežnu funkciju, dok je u drugim stadijumima, vremenom, progresivno opadala funkcija bubrega. Bubrežna funkcija najsporije je opadala kod

cose, as well as the frequency of erythrocyturia, did not differ statistically significantly between the compared groups (Table 2; Table 3).

Patients in whom NCAM-expressing interstitial cells were detected had statistically significantly lower proteinuria levels at the time of biopsy, as compared to patients whose biopsy samples did not have NCAM-positive cells in the interstitium (3.97 g/24 h vs. 8.41 g/24 h; $p = 0.024$), (Table 2).

The erythrocyte count, hemoglobin level, hematocrit level, and the mean corpuscular volume (MCV) did not differ significantly between the compared groups (Table 2; Table 3). Patients were followed up for an average of 16 months. Pathohistological and clinical features were observed as potential predictors of CKD progression to the advanced stages. We observed that none of the patients with minimal changes progressed to advanced stages of CKD during the follow-up period, while all patients with rapidly progressive glomerulonephritis (GN) had a rapid deterioration of renal function. However, due to the large number of different pathohistological diagnoses and the relatively small



Slika 2. Verovatnoća pogoršanja funkcije bubrega u zavisnosti od (A) vrednosti serumskog kreatinina, (B) uree i (C) proteinurije

Figure 2. Probability of renal function deterioration depending on (A) serum creatinine level, (B) urea level, and (C) proteinuriae

pacijenata sa početnom intersticijskom fibrozom, brže u drugom stadijumu, dok je u trećem najbrže dolazilo do stradanja bubrežne funkcije. Međutim, zbog malog broja pacijenata uključenih u studiju preživljavanja, a uz gradiranje stepena fibroze na četiri stadijuma, statistička analiza nije urađena.

Verovatnoća pogoršanja bubrežne funkcije je značajno zavisila od vrednosti serumskog kreatinina ($p < 0,001$) i uree ($p = 0,007$). Bubrežna funkcija je znatno duže bila očuvana kod normalnih vrednosti serumskog kreatinina (< 104 *μmol*/l, referentna vrednost laboratorije Univerzitetskog kliničkog centra Srbije), (Slika 2. A) i uree (< 7,5 mmol/l, referentna vrednost laboratorije Univerzitetskog kliničkog centra Srbije), (Slika 2. B), za razliku od pacijenata koji su imali povišene vrednosti *sCr* i uree, gde je dolazilo do rapidnog pogoršanja bubrežne funkcije. Primetili smo da su pacijenti koji su imali proteinuriju manju od 3 g/24 h značajno duže održavali bubrežnu funkciju, u odnosu na pacijente koji su imali proteinuriju preko 3 g/24 h. Međutim, proteinurija nije bila značajan prediktor lošeg ishoda bolesnika ($p = 0,231$), kao što je prikazano na Slici 3. C.

DISKUSIJA

Intersticijska fibroza se javlja kod etiološki različitih oboljenja bubrega. Napredovanje intersticijske fibroze dovodi do progresivnog gubitka bubrežne funkcije, dovodeći pacijente do potrebe za dijalizom i transplantacijom bubrega [12]. Sve je više studija koje ispituju molekularnu osnovu fibroze, a među najvažnijima su one koje su fokusirane na ispitivanje ranih pokazatelja potencijalne progresije bolesti.

Fibroza predstavlja abnormalnu akumulaciju ekstracelularnog matriksa. Smatra se da različite ćelije mogu doprineti produkciji ekstracelularnog matriksa, ali da su glavne ćelije aktivirani fibroblasti, tj. miofibroblasti [12,13]. Poznato je da su aktivacija i mobilizacija fibroblasta rezultat stimulacije receptora 1 fibroblastnog faktora rasta (engl. *fibroblast growth factor receptor-1 – FGFR-1*). *FGFR-1* može biti stimulisan različitim ligandima uključujući i *NCAM* [14,15,16], što nas je

number of patients in these groups, statistical analysis was not performed.

It was also observed that patients without interstitial fibrosis, during the three years of follow-up, preserved renal function in 90% of cases, while in other stages, renal function progressively declined over time. Renal function declined the slowest in patients with incipient interstitial fibrosis, more rapidly in the second stage, while in the third stage, kidney function declined the fastest. However, due to the small number of patients included in the survival study and the degree of fibrosis (graded into four stages), statistical analysis was not performed.

The probability of renal function deterioration was significantly dependent on the serum creatinine level ($p < 0.001$) and the urea level ($p = 0.007$). Renal function was preserved for a significantly longer period of time with normal levels of serum creatinine (< 104 μmol/l, reference value of the laboratory of the University Clinical Center of Serbia), (Figure 2. A) and normal levels of urea (< 7.5 mmol/l, reference value of the laboratory of the University Clinical Center of Serbia), (Figure 2. B), as opposed to patients who had elevated levels of sCr and urea, in whom there was a rapid deterioration of renal function. We observed that patients who had proteinuria below 3 g/24 h maintained kidney function significantly longer, as compared to patients who had proteinuria above 3 g/24 h. However, proteinuria was not a significant predictor of poor patient outcome ($p = 0.231$), as shown in Figure 2. C.

DISCUSSION

Interstitial fibrosis occurs in etiologically different kidney diseases. Progression of interstitial fibrosis leads to progressive loss of renal function (kidney failure), leading to patients requiring dialysis and kidney transplantation [12]. There is an increasing number of studies examining the molecular basis of fibrosis, and among the most important are those focused on examining early indicators of potential disease progression.

navelo da detaljnije ispitujemo ekspresiju *NCAM* molekula na intersticijskim ćelijama u različitim stadijumima intersticijske fibroze.

U intersticijumu zdravih bubrega, *NCAM* eksprimirajuće ćelije su vrlo retke, međutim tokom određenih patoloških stanja njihov broj može da se poveća [17]. Rezultati našeg istraživanja pokazali su da se veći broj *NCAM* eksprimirajućih ćelija gotovo isključivo pojavljuje u početnim fazama intersticijske fibroze, kod etiološki različitih oboljenja bubrega, dok je u uznapredovalim stadijumima ekspresija znatno niža. Rezultati drugih istraživača sugerisali su da je obim *NCAM* ekspresije u intersticijumu usko povezan sa stepenom oštećenja intersticijuma, kako kod čoveka, tako i kod životinja [4,18]. Tako je primećeno naglo povećanje *NCAM* ćelija u ranoj fazi procesa regeneracije nakon ishemijskog oštećenja tubula kod pacova [19]. Takođe, Luo i saradnici su dokazali značajnu zastupljenost *NCAM*-a u odmaklim stadijumima fibroze jetre, kod pacijenata sa nealkoholnom steatozom jetre [20]. U *in vitro* modelu intersticijske fibroze bubrega, uočena je snažna indukcija ekspresije izoformi *NCAM*-a zajedno sa *FGFR1* receptorom, i to 24 sata nakon stimulacije procesa fibrogeneze pod uticajem transformišućeg faktora rasta beta 1 (engl. *transforming growth factor beta – TGF-β1*), iako u tom trenutku morfološke promene nisu bile vidljive. Nakon 48 sati, primećen je pad nivoa *NCAM* i *FGFR1* mRNA, što ukazuje na ključnu ulogu ovih molekula u inicijaciji procesa fibrogeneze. Takođe, blokiranjem *FGFR1* signalnog puta nakon izlaganja *TGF-β1*, uočena je morfološka reverzija procesa fibrogeneze, čime je potvrđena značajna uloga *NCAM/FGFR1* u inicijalnoj fazi fibroze bubrega [10] . Otkrivanje molekularnih mehanizama fibrogeneze, važnih u fazi inicijacije fibroze, značajno je zbog primene novih, ciljanih terapijskih modaliteta, čime bi se smanjila potreba za dijalizom i transplantacijom bubrega kod pacijenata obolelih od različitih, etiološki heterogenih, netumorskih oboljenja bubrega.

Ekspresija *NCAM*-a, kao patološki supstrat, osim u fibrozi bubrega, prepoznata je i u srcu, plućima i hepatobilijarnom sistemu. Studije takođe ukazuju na potencijalni razvoj novih terapijskih modaliteta u cilju usporavanja progresije fibroze, i sugerišu na mogućnost reverzije procesa fibrogeneze [21–25].

Naša studija se jedina bavila ispitivanjem kliničkog značaja detekcije *NCAM* ćelija u intersticijumu bubrega nefroloških bolesnika, pri čemu je ustanovljeno da su pacijenti sa *NCAM* eksprimirajućim ćelijama u intersticijumu imali znatno niže prosečne vrednosti proteinurije. Zbog pretpostavke da postoji mogućnost da *NCAM* eksprimirajuće intersticijske ćelije predstavljaju zapravo eritropoetin

Fibrosis is an abnormal accumulation of extracellular matrix. It is believed that different cells can contribute to the production of extracellular matrix, but that the main cells in this process are activated fibroblasts, i.e., myofibroblasts [12,13]. It is known that the activation and mobilization of fibroblasts is the result of the stimulation of fibroblast growth factor receptor 1 (FGFR-1). FGFR-1 can be stimulated by different ligands including NCAM [14,15,16], which has led us to examine in more detail the expression of NCAM molecules in interstitial cells, in different stages of interstitial fibrosis.

In the interstitium of healthy kidneys, NCAM-expressing cells are very rare, but during the course of certain pathological conditions their number can increase [17]. The results of our study showed that a greater number of NCAM-expressing cells almost exclusively appears in the initial stages of interstitial fibrosis, in etiologically different kidney diseases, while in advanced stages the expression is significantly lower. The results of other researchers suggested that the extent of NCAM expression in the interstitium is closely related to the degree of interstitial damage, both in humans and animals [4,18]. Thus, a sudden increase of NCAM cells was observed in the early phase of the regeneration process after ischemic tubule damage in rats [19]. Also, Luo et al proved a significant presence of NCAM in advanced stages of liver fibrosis, in patients with non-alcoholic steatosis of the liver [20]. In an in vitro model of kidney interstitial fibrosis, a strong induction of the expression of NCAM isoforms together with FGFR1 was observed, 24 hours after stimulation of the fibrogenesis process under the influence of transforming growth factor beta 1 (TGF-β1), although, at that moment, morphological changes were not visible. After 48 hours, a decrease in NCAM and FGFR1 mRNA levels was observed, indicating the key role of these molecules in the initiation of the fibrogenesis process. Also, by blocking the FGFR1 signaling pathway after exposure to TGF-β1, morphological reversion of the fibrogenesis process was observed, thus confirming the significant role of NCAM/FGFR1 in the initial stage of kidney fibrosis [10]. The discovery of molecular mechanisms of fibrogenesis, important in the initial phase of fibrosis, is significant due to the application of new, targeted therapeutic modalities, which would reduce the need for dialysis and kidney transplantation in patients suffering from various, etiologically heterogeneous, non-tumor kidney diseases.

The expression of NCAM, as a pathological substrate, apart from kidney fibrosis, has also been found in the heart, the lungs, and the hepatobiliary system. Studies also indicate the potential development of

produkujuće ćelije [7], ispitivali smo odnos distribucije pacijenata koji eksprimiraju *NCAM* ćelije i vrednosti eritrocita, hemoglobina, hematokrita i srednje zapremine eritrocita, međutim, nismo uočili povezanost.

Studije koje se bave određivanjem kliničkih i patoloških prediktora pogoršanja bubrežne funkcije imaju veliki praktični značaj. Među kliničkim parametrima, značajni prediktori pogoršanja funkcije bubrega su povišene vrednosti kreatinina i uree u serumu pacijenata, kao i visoke vrednosti proteinurije, naročito one nefrotskog ranga [19,26,27]. Među ispitivanim kliničkim parametrima u našoj studiji, kao prediktori pogoršanja bubrežne funkcije takođe su se izdvojile povišene vrednosti serumskog kreatinina i uree. U dosadašnjim istraživanjima je uočeno da proteinurija predstavlja prediktor lošeg ishoda [26,28,29]. Mi smo primetili da pacijenti koji su imali proteinuriju manju od 3 g/24 h, duže održavaju normalnu bubrežnu funkciju, međutim, proteinurija se nije izdvojila kao značajan prediktor pogoršanja bubrežne funkcije. Poznato je i da verovatnoća progresije bolesti bubrega u uznapredovale *CKD* stadijume značajno zavisi od patohistološke dijagnoze i stadijuma intersticijske fibroze [30], što zbog malog broja pacijenata u našoj studiji nije statistički ispitivano.

ZAKLJUČAK

Prisustvo *NCAM* ćelija u intersticijumu bubrega je karakteristika ranih faza hroničnih bolesti bubrega sa početnom intersticijskom fibrozom i blažim stepenom proteinurije. Vrednosti serumskog kreatinina i uree značajno su uticale na verovatnoću pogoršanja funkcije bubrega, te predstavljaju važne kliničke prediktore ulaska pacijenata u uznapredovale *CKD* stadijume.

Sukob interesa: Nije prijavljen.

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new therapeutic modalities in order to slow down the progression of fibrosis and suggest the possibility of reversing the process of fibrogenesis [21–25].

Our study was the only one to examine the clinical significance of the detection of NCAM cells in the kidney interstitium of nephrological patients, and it was established that patients with NCAM-expressing cells in the interstitium had significantly lower average proteinuria levels. Because of the assumption that there is a possibility that NCAM-expressing interstitial cells actually represent erythropoietin producing cells [7], we examined the relationship between the distribution of patients expressing NCAM cells and the erythrocyte count, the hemoglobin level, the hematocrit level, and the mean corpuscular volume (MCV). However, we did not observe any association.

Studies focused on determining clinical and pathological predictors of renal function deterioration are of great practical importance. Among the clinical parameters, significant predictors of the deterioration of kidney function are elevated levels of creatinine and urea in the serum of patients, as well as high levels of proteinuria, especially those in the nephrotic range [19,26,27]. Among the examined clinical parameters in our study, elevated levels of serum creatinine and urea also stood out as predictors of renal function deterioration. In previous studies, it has been observed that proteinuria is a predictor of poor outcome [26,28,29]. We observed that patients who had proteinuria below 3 g/24 h maintained normal renal function longer, however, proteinuria did not stand out as a significant predictor of declining renal function. It is also known that the probability of kidney disease progression to advanced CKD stages significantly depends on the pathohistological diagnosis and stage of interstitial fibrosis [30], which was not statistically investigated due to the small number of patients in our study.

CONCLUSION

The presence of NCAM cells in the renal interstitium is characteristic of the early stages of chronic kidney disease with incipient interstitial fibrosis and mild proteinuria. The levels of serum creatinine and urea significantly affected the probability of kidney function deterioration, which is why they represent important clinical predictors of patients progressing to advanced stages of CKD.

Conflict of interest: None declared.

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