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ORIGINAL ARTICLE

Immunohistochemical expression of proliferative markers in renal cell carcinoma

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Summary

Purpose: *The purpose of this study was to investigate into the expression of cyclin A and telomerase in renal cell carcinoma (RCC) and to analyze the relationship between expression and the clinicopathological characteristics of the tumor and their impact on survival.*

Methods: *The overall material included 74 samples of RCC and 4 of normal renal tissue. Primary cyclin A antibody from Santa Cruz Biotechnology and TERT MA5-16034 antibody from Thermo Fisher Scientific Inc were used. Staining was performed by streptavidin-biotin technique using DAKO LSAB+ kit. Statistical analyses were performed using of SPSS 23 Statistics software from IBM.*

Results: *No differences in cyclin A and telomerase expression among gender and age groups were found, nor did the tumor dimensions have any significant impact on expression. Also, tumor grades and stages did not differ. However, histological types differed in favor of the papillary type. A significant positive correlation between both markers, as well as between the expression and tumor stage and grade was noticed. Only the tumor stage had negative impact on survival.*

Conclusions: *Although not affecting survival, the expression of cyclin A and telomerase increased with tumor stage and grade, suggesting that cyclin A and telomerase could be potential proliferative immunohistochemical markers of RCC.*

Key words: *cyclin A, proliferative markers, renal cell carcinoma, survival, telomerase*

Introduction

RCC is the prevailing form of renal cancer in adults, 14th most common cancer worldwide and accounts for approximately 3% of all cancer diagnoses [1-4]. Due to its large resistance to chemotherapy and radiotherapy, RCC is a very aggressive and often fatal disease. Partial or complete response to immunotherapy is noticed only in a small proportion of patients, due to absence of specific tumor antigens [5-8]. The typical onset of RCC is between 40-70 years of age with male predominance [3,9,10]. In recent years, its incidence shows signs of plateauing or decrease [2]. Nevertheless, due to lack of early symptoms and signs, the majority of RCC are discovered incidentally via imaging studies, where 25% of patients already have advanced disease with 5-year survival of 10% [11].

Today's recognized clinicopathological tools used to improve the predictability of RCC, such as TNM classification, vascular invasion, necrosis and the Fuhrman nuclear grading are still insufficient [5,6]. Many studies have shown that Fuhr-

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man nuclear grading, which has been accepted by the majority of pathologists worldwide, is affected by substantial intra-observer and inter-observer variations. Because it relies solely on nuclear pleomorphism, size and prominence, there are no clear guidelines for cases which do not fit in any category [7]. A recent study has suggested that Fuhrman nuclear grading has no prognostic importance for chromophobe types [8] , while only nuclear pleomorphism has prognostic significant in papillary types [9].

Therefore, there is a need for a prognostic marker that might act as substitute for Fuhrman nuclear grade and also be more objective in its interpretation. Significant advances in molecular medicine have given insights into the molecular alterations and subsequent downstream pathways concerning tumorigenesis and tumor progression. Promising prognostic markers have already been identified and they include indicators of cell proliferation, cell adhesion, and indicators directly associated with cell growth regulation, all of which can be detected via assays like immunohistochemistry and used to assess the biological behavior of the tumor [10-14]. Understanding this is principal in order to raise the potential of predicting the outcome and response to systemic therapies, especially in the era of targeted therapies [15-17].

Cyclins are primary cell cycle-specific regulators controlling its major checkpoints [18,19]. A redundant pattern of expression is found virtually in all tumor cells, making cyclins acting as proto-oncogenes [18]. An association of cyclin A abnormalities with carcinogenesis has also been noticed [20]. Cyclin A regulates multiple steps of the cell cycle. It is mandatory for DNA replication throughout the S-phase and in complex with CDK2 represents rate-limiting component which is required for cell entry and progression through mitosis [18,21,22]. According to this, overexpression of cyclin A is an unfavorable prognostic factor, as shown in the case of RCC, soft-tissue sarcomas, breast cancer and in non-small-cell lung cancer [10,23-25].

Telomerase represents a ribonucleoprotein complex required for chromosomal stability [26, 27]. It consists of human telomerase reverse transcriptase (hTERT), a catalytic protein which replicates the ends of linear DNA, and intrisic human telomerase RNA (hTR) which serves as a base template for replication [28]. It prevents critical consequences of exposed DNA ends, such as chromosomal end-to-end fusions and nucleolytic processing, thus providing solution to the end-replication problem [29]. The activity of telomerase is normally inhibited during the embryonic period, but remains active in germinative cells and stem cells of various tissues. It is also present in immortalized cells as well as in practically all human tumors, but not in normal adjacent cells [26,27,30]. Telomere stability is required for long-term proliferation, so by activating telomerase tumors can escape cellular senescence and become immortal [31-33].

The aim of this study was to investigate the expression of cyclin A and telomerase in RCC, and to analyze the relationship between expression and the clinicopathological characteristics of the tumor (histopatological type, Fuhrman nuclear grade, tumor stage), and their impact on survival.

Methods

The operative material used in our study was obtained after partial nephrectomy performed at the Clinic of Urology of the Clinical Center of Serbia and at the Clinic-hospital Center "Dr Dragisa Misovic". Both Ethics Committees gave their approval. The diagnosis was made at the Institute of Pathology of the Belgrade School of Medicine.

The entire material consisted of 74 samples of RCC which were prepared using a standard method, and 4 samples of normal renal tissue. To determine the clinicopathological charateristics of the tumor the WHO classification of 2004 and the AJCC cancer staging manual were used [34,35].

The treatment of samples involved antigen unmasking by citrate buffer at pH 6.0 in a microwave oven, 3 cycles of 5 min. Endogenous peroxidase activity was blocked by 3% hydrogen-peroxide during 5 min. In order to reduce nonspecific staining pork serum in a dilution of 1:10, for 30 min was used. We applied primary cyclin A antibodies from Santa Cruz Biotechnology, CA, USA, in a dilution of 1:200, and TERT MA5-16034 antibodies from Thermo Fisher Scientific Inc., Invitrogen Waltham, MA, USA, in a dilution of 1:50, both for 60 min. Staining was performed by streptavidin-biotin technique using LSAB+ kit (DAKO Cytomation, Glostrup, Denmark). 3,3-diaminobenzidine was used as chromogen and Mayer's hematoxylin was used for contrast staining.

Immunohistochemical staining was evaluated semiquantitatively. Samples with moderate (10-50% stained cells) and diffuse expression (>50% stained cells) were considered as positive, while the ones with focal expression (<10% stained cells) or with an absence of staining were considered as negative.

Statistics

Statistical analyses were performed using the SPSS 23 Statistics software from IBM. We used median values + range and mean values ±SD for quantitative variables such as patient age, tumor dimensions and expression level. The x^2 test was used to compare different groups. The Kruskall-Wallis test was used to determine a correlation of cyclin A and telomerase with tumors' stage and

grade. Pearson's correlation test was used to determine the correlation between both markers and Spearman's rank test was used to determine the relationship of both markers in the semi-quantitative analysis of expression. To assess the relationship between expression and clinicopathological characteristics with survival we used Kaplan-Meier method and Cox- multivariate regression analysis. All p values were two-tailed and values less than 0.05 were considered significant.

Results

In normal renal parenchyma adjacent to the tumor, focal expression of cyclin A was found in epithelial cells of distal convoluted tubules. Telomerase was not detected in any sample. Therefore, moderate and diffuse expressions were considered as overexpression.

Table 1. Clinicopathological data

Clinicopathological data	*n*	*%*
Histopatological type		
Clear cell	49	66.2
Papillary	18	24.3
Chromophobe	7	9.5
Fuhrman nuclear grade		
1	4	5.4
2	37	50
3	30	40.6
4	3	4
Tumor stage		
I	29	39.2
II	12	16.2
III	30	40.6
IV	3	4

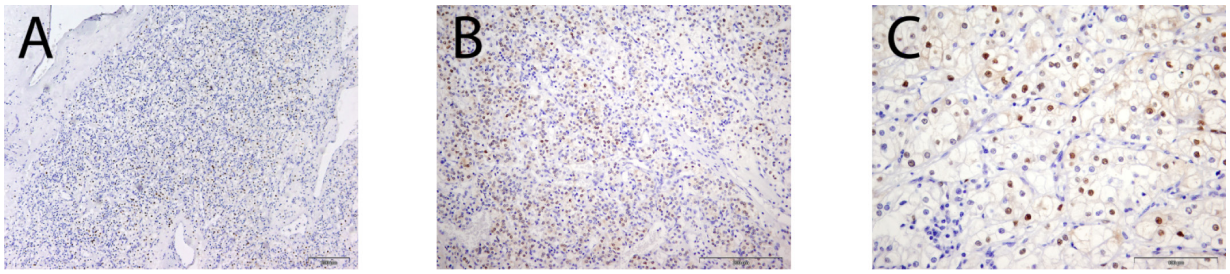

Figure 1. A: Moderate expression of cyclin A in a papillary type of RCC at ×200 magnification; **B:** Diffuse expression of telomerase in a papillary type of RCC at ×200 magnification; **C:** Diffuse expression of telomerase in a clear cell type of RCC at ×400 magnification.

Table 2. The expression of cyclin A and telomerase in RCC*

	Samples n (% of total)	*Cyclin A positive samples n (% of positive)*	*p value*	*Telomerase positive n (% of positive)*	*p value*
Gender			0.316		0.957
Male	48 (64.9)	20 (41.7)		28 (58.3)	
Female	26 (35.1)	14 (53.8)		15 (57.7)	
Age group, years**			0.472		0.970
<60	36 (48.6)	15 (41.7)		21 (58.3)	
>60	38 (51.4)	19 (50.0)		22 (57.9)	
Tumor dimensions, mm**			0.072		0.387
<48	41 (55.4)	15 (36.6)		22 (53.7)	
>48	33 (44.6)	19 (57.6)		21 (63.6)	
Histopathological type			<0.001		<0.001
Clear cell	49 (66.2)	16 (32.7)		23 (46.9)	
Papillary	18 (24.3)	16 (88.9)		18 (100.0)	
Chromophobe	7 (9.5)	2 (28.6)		2 (28.6)	
Fuhrman nuclear grade ***			0.69		0.934
Lower	41 (55.4)	18 (43.9)		24 (58.5)	
Higher	33 (44.6)	16 (48.5)		19 (57.6)	
Tumor stage ****			0.94		0.934
Lower	41 (55.4)	19 (46.3)		24 (58.5)	
Higher	33 (44.6)	15 (45.5)		19 (57.6)	

*Values represent the number (percentage) of positive samples (moderate and diffuse expression). **Groups were formed according to the mean value of the variable. ***Lower grades I and II; Higher grades III and IV. ****Lower stages I and II; Higher stages III and IV.

Patient age ranged between 33 and 85 years (mean of 59.27±10.3). Out of 74 samples, 48 (64.9%) originated from male patients and 26 (35.1%) from female patients (male-to-female ratio of 2:1). Tumor dimensions varied between 15 and 130 mm (mean 48.30±23.47). Other clinicopathological data are summarized in Table 1.

Cyclin A was present in 34 (45.9%) samples and telomerase was present in 43 (58.1%) samples, with mean expression level of 16.8±18.6% (median=10) and 24.9±22.7% (median=20), respectively (Figure 1).

There were no differences in expression with regard to gender and age, nor did the tumor dimensions have any significant impact on expression. Also, tumor grades and stages did not differ. However, histological types differed favoring the papillary type, where almost all papillary type samples expressed cyclin A and all were positive for telomerase. Also, the expression level was significantly lower in clear cell and chromophobe types compared to the papillary type. Data are summarized in Tables 2 and 3.

Using Pearson's correlation and Spearman's Rank-Order Correlation tests we determined that there was a significant positive correlation of r=0.732 and ρ=0.563 between both markers at p<0.01 (Figure 2). The expression of cyclin A and telomerase increased with grade and stage, although no significant relationship was found (Table 3).

We observed that only tumor stage had negative impact on survival (p<0.001), unlike cyclin A and telomerase expression, patient gender and age, tumor dimensions, histopatological type and Furhman nuclear grade which had none (Table 4, Figures 3-5).

Table 3. Expression levels of cyclin A and telomerase in RCC*

	Cyclin A	*Telomerase*
Histopatological type		
Clear cell	11.1±15.3	15.3±14.4
Papillary	35.3±17.5	56.1±14.8
Chromophobe	8.7±11	12.1±11.9
Fuhrman nuclear grade		
I	3.75±7.5	15±7
II	15.3±16.7	22.8±20.8
III	19±21.3	29.7±26.5
IV	30±17.3	16.7±5.7
Tumor stage		
I	12.6±16.7	20.5±21.2
II	27.5±22.8	40.0±28.4
III	13.5±15	22.5±20.8
IV	46.67±15.3	31.7±10.4

*Values are mean±SD

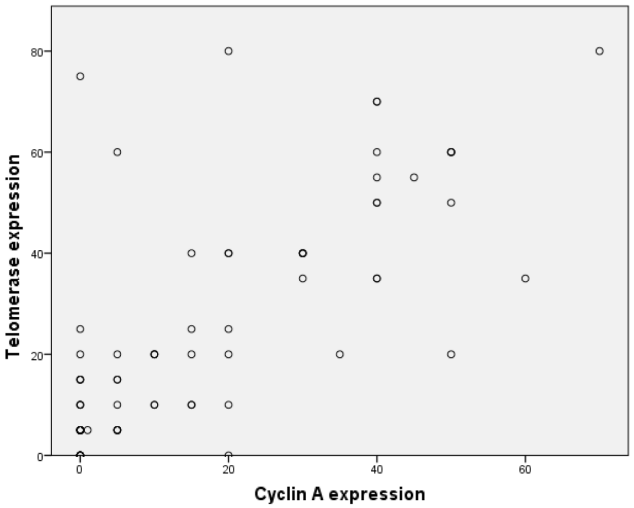

Figure 2. Scatter plot chart showing positive correlation among cyclin A and telomerase expression.

Table 4. Survival in RCC

				95% CI for hazard ratio	
	Coefficient	*p value*	*Hazard ratio*	*Lower*	*Upper*
Gender	0.146	0.584	1.157	0.651	2.055
Age	-0.146	0.619	0.864	0.513	1.456
Tumor size	-0.123	0.640	0.884	0.528	1.481
Histopathological type	0.155	0.430	1.168	0.794	1.719
Tumor stage	0.947	0.001	2.577	1.465	4.533
Fuhrman nuclear grade	0.062	0.812	1.064	0.639	1.772
Cyclin A expression	0.098	0.755	1.103	0.597	2.038
Telomerase expression	-0.188	0.555	0.829	0.444	1.546

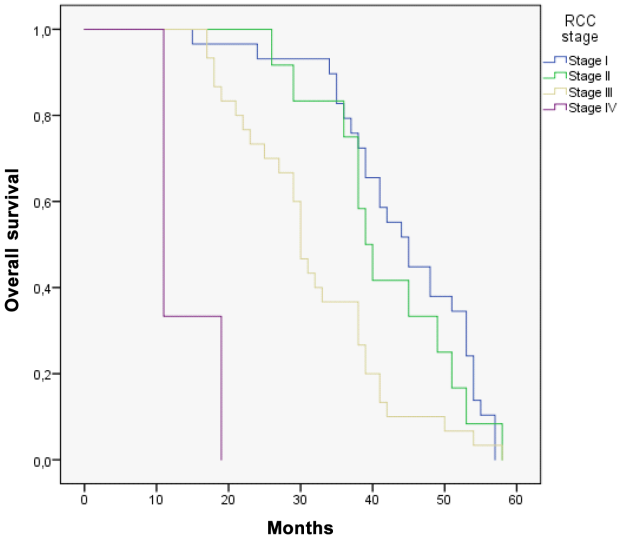

Figure 3. Overall survival among different stages of RCC. Survival was significantly shorter in advanced stages (p<0.001).

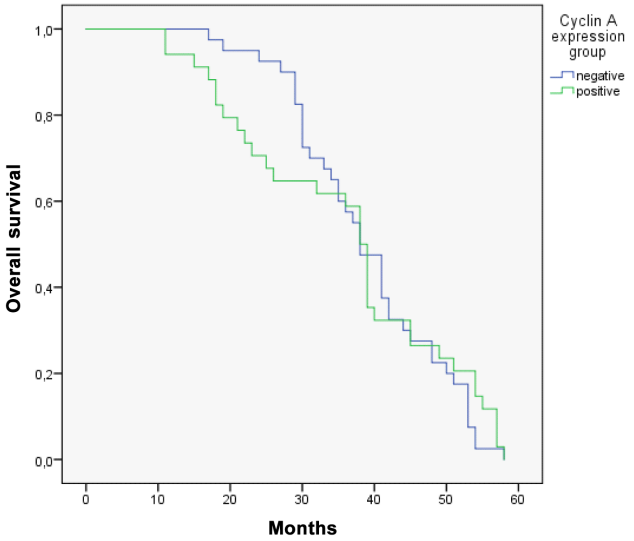

Figure 4. Overall survival among cyclin A positive and negative groups. No significant difference in survival between cyclin A positive and negative patients was noticed (p=0.497).

Discussion

RCC has the worst prognosis of all the urological tumors. The tumor stage remains the most powerful predictor of prognosis, as it was shown in our study. The Fuhrman nuclear grading is still considered the most important prognostic parameter among histopatological parameters. Our study dealt mainly with lower grade tumors as 55.4% were grade I or II, which was similar to the study conducted by Latif et al., where around 66% of the tumors were grade II [36], and in contrast to Frank et al., who found that 46.6% tumors were either grade III or IV [37].

There is only some similarity in the percentage of tumors within each grade, because it is difficult to observe all the criteria of Fuhrman nuclear grading, so the pathologist is often left with his own interpretation of the system. The Fuhrman nuclear grading seems to have no value in the chromophobe type, while papillary types are graded better using only the criteria of nucleolar prominence [8,9].

Proteins involved in driving the cell cycle, such as cyclins, are often overexpressed in primary tumors as a result of gene amplification, chromosomal translocation or dysregulated expression. Increased expression of cyclin A has been demonstrated in a number of studies [10,38-44] among which the one conducted by Volm et al., concerning non-small-cell lung cancer, compared the survival of patients with negative and positive cyclin A staining in their primary tumor, where those with positive staining had a worse outcome compared with those with negative staining [44]. In contrast, Wang et al. reported that the cyclin A expression in normal colon mucosa was higher than in cancer tissue in 63% of the cases, and only 10% of cancers had a higher cyclin A expression [45]. Also, Kim et al. found increased cyclin A expression in hyperplastic skin and in benign papillomas, however, the expression decreased in squamous cell carcinoma [46].

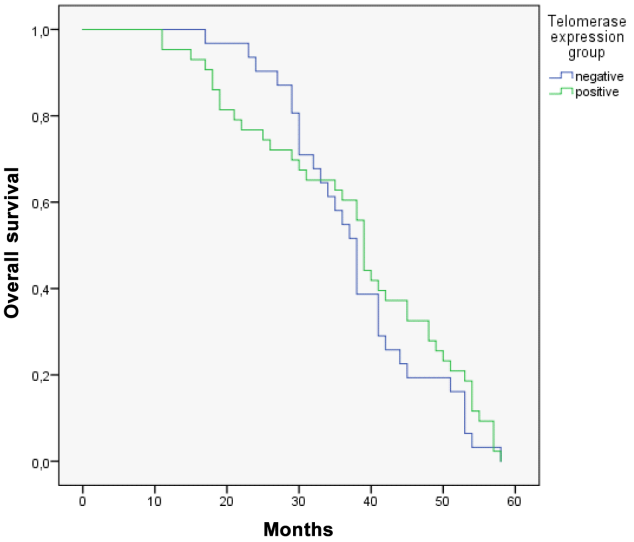

Figure 5. Overall survival among telomerase positive and negative groups. No significant difference in survival between telomerase positive and negative patients was noticed (p=0.894).

Although the relationship between tumor stage and cyclins is obvious in other types of carcinomas [10,38-41], in our study we could not find

any. Also, no significant association between cyclin A expression and gender, age, tumor size or grade was found. Only histological types of RCC differed, where the papillary type dominantly expressed cyclin A. Cyclin A expression did not correlate with survival.

The majority of studies rely on immunohistochemical detection of cyclin A. However, the important question of whether elevation of cyclin A is a contributing factor to tumorigenesis or a mere consequence of increased cell proliferation is not easily addressed. Not surprisingly, cyclin A is typically coexpressed with proliferation markers such as PCNA (proliferative cell nuclear antigen) and Ki67. Despite these limitations, expression of cyclin A in many types of cancers appears to be of prognostic value such as prediction of survival or early relapse.

The telomerase activity has been found in a variety of malignant tumors and in most cell lines, including RCC, in contrast to normal somatic tissues or cell strains where it has not been detected [47-50]. Telomerase is required for long-term proliferation and thus important for the growth and development of cancer, making it a parameter worth researching. Our study demonstrated that 58.1% of the samples were positive for telomerase activity. The positive frequency was somewhat lower than reported by others [51], who found no obvious association between positive telomerase activity and clinicopathological parameters. We were also unable to find any significant association between telomerase activity and gender, age, tumor size, grade or stage. Several reports have cited a gap in the frequency of telomerase activity according to tumor subtype. In lung cancer, almost 100% of small-cell carcinoma were telomerase-positive, in contrast to 78% of non-small-cell carcinomas [52]. Kinoshita et al. reported that 17% of chromophobe RCC type showed positive telomerase activity, as opposed to 93% of clear cell RCC and 85% of all RCC [51]. In this study, we concluded that the papillary type dominantly expressed telomerase given that all the samples were positive on telomerase. Telomerase activity also did not have an impact on survival.

In conclusion, although not affecting survival, the expression of cyclin A and telomerase increased with tumor stage and grade. Also, almost all papillary type samples expressed cyclin A and all were positive for telomerase, suggesting that cyclin A and telomerase could be potential proliferative immunohistochemical markers of RCC.

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Conflict of interests

The authors declare no conflict of interests.

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