Immunohistochemical study of cyclin A and p16 expression in patients with renal cell carcinoma

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Summary

Purpose: Renal cell carcinoma (RCC) is the most common malignant kidney tumor in adults. Dysregulation of the cell cycle can lead to cancer development. In this study, the mitosis-associated cyclin A and p16, a negative controller, were investigated as potential key points in the RCC development.

Methods: This retrospective study included 74 patients with RCC. The expression of cyclin A and p16 and their correlation to histopathological parameters (TNM stage, histological subtype, nuclear grade, tumor size), gender, age, and clinical outcome were studied and analyzed.

Results: The highest median value for cyclin A (40% ; range 0-70)) and for p16 (57.5% ; range 35-80) were found in the papillary histological subtype. Survival analysis showed that in the group of patients that had died before September 2015, the median value for cyclin A was 20% (range 0-60), which was significantly higher than 5% (range 0-70), found in the group of patients that survived (p=0.019).

Conclusions: In relation to the histological subtype, the papillary type of RCC was associated with a significantly higher expression of cyclin A and p16 compared to other subtypes of RCC. High expression of cyclin A indicated worse prognosis, therefore cyclin A could be considered to be a significant prognostic marker.

Key words: cyclin A, immunohistochemistry, p16, renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) is the most common malignant kidney tumor in adults. It is usually sporadic, and rarely (<4% of kidney tumors) it is associated with some familial syndrome, occurring at an earlier age, often bilateral, and multicentered [1].

Stage on presentation, low nuclear grade and multilocular cystic histological type of RCC were found to have the best prognosis, whereas collecting duct RCC has the worst prognosis [2]. It is possible to find dedifferentiations within each histological type of RCC (malignant fibrous histiocytoma, rhabdomyosarcoma, angiosarcoma) [3]. Sarcomatoid dedifferentiation, which can develop in all types of RCC, represents malignant transformation of higher grade and is associated with poor prognosis [4,5]. Some patients with encouraging prognostic parameters develop metastatic disease and have adverse outcome. It is, therefore, necessary to find a molecular prognostic parameter that will accurately determine the proliferative activity of RCC.

Cyclin A is the only described double-role cyclin in the cell cycle. It is essential for the replication of DNA during S phase, and has an active role in the initiation of mitosis [6,7]. Overexpression of cyclin A was observed in a large number of human tumors (lung cancer, breast cancer, colon cancer, transitional cell carcinoma of the kidneys and the ureters and ovarian cancer with longer survival [8]), and it correlates with unfavorable prognostic factors. Usually there is a co-expression of cyclin A with proliferative markers, such as PCNA and Ki67 [9,10].

Tumor suppressor protein p16 (cyclin-dependent kinase inhibitor 2A) is an inhibitor of CDK4 and CDK6. The cellular expression of p16 is highly selective and it does not correlate with cell proliferation and maturation. The level of p16 dramatically increases with the ageing of cells. In adults, p16 is normally expressed in the proliferative endometrium, breast ductal and esophageal epithelium, epithelium of the cervix and endocrine glands. In children, the expression of p16 is limited to HASAL body and, rarely, thymic lymphocytes and epithelial cells of the pancreas [6]. P16 gene (CDKN2A) is the most commonly inactivated tumor suppressor gene in pancreatic cancer (95% of the patients) [11].

We found several studies of the cell cycle regulation in RCC [12-14]. In this study, the role of cyclin A (in the phase of mitosis), and p16 (as a negative cyclin D controller, which if present in a sufficient amount starts the cell cycle) as potential key points in RCC proliferation has been analyzed. The aim of this study was to determine the immunohistochemical (IHC) expression of cyclin A and p16 in patients with RCC and to correlate it to clinical and morphological parameters (TNM stage, nuclear grade, histological type, tumor size, survival).

Methods

This retrospective study included 74 patients with RCC who were operated in the Clinical Hospital Center "Dragisa Misovic-Dedinje" and the Clinic of Urology, Clinical Centre of Serbia. They were diagnosed at the Institute of Pathology, School of Medicine in Belgrade during a period of 4 years. All procedures were approved by the Ethics Committee of the Clinical Hospital Centre "Dr Dragisa Misovic" and the Clinical Centre of Serbia in accordance with the Declaration of Helsinki. Clinical data (gender, age, tumor size, clinical outcome at the time of the study conclusion) and histopathological parameters (TNM stage, nuclear grade, histological type) were collected for each patient. Of the 74 patients, 48 (64.86%) were males and 26 (34.14%) females (Figure 1), and the mean age at the operation was 59.29
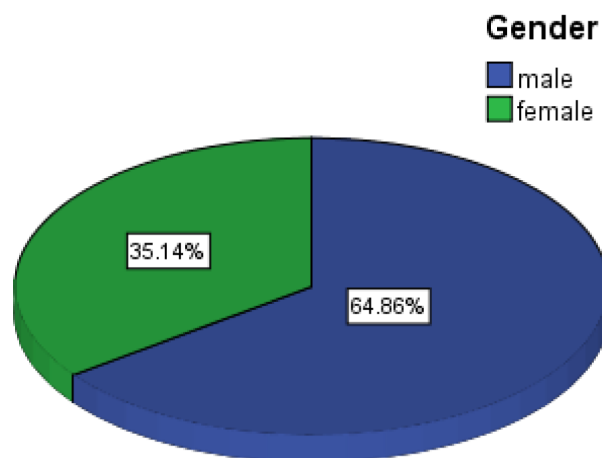

Figure 1. Patients with RCC by gender.

years (range 33-85). Tumor stage, histological type and prognostic group were determined according to the 2004 WHO classification. Accordingly, 41 (55.41%) out of 74 cases were classified as grade I/II (low grade) and 33 (44.59%) as grade III/IV (high grade). Stages pT1 and pT2 were classified as low-stage tumors - 42 out of 74 patients (56.76%). pT3 and pT4 were high-stage tumors (32 patients;43.24%). Analysis of the different histological types of RCC showed clear cell (49 cases; 66.22%), chromophobe (7 patients; 9.46%), papillary (18 patients; 24.32%) and collecting duct (0) disease.

Immunohistochemistry

Rabbit polyclonal antibodies to cyclin A and p16 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sections (5-nm thick) from the formalin-fixed, paraffin-embedded tissue samples were deparaffinized and treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. For the heat-induced antigen retrieval, tissue sections were immersed in 0.01mol/ L^{-1} citrate buffer (pH=6.0) and treated in a microwave oven for 20 min at 620 W. After cooling off for 30 min at room temperature, blocking peptide (DAKO, Glostrup, Denmark) was utilized to block the non-specific staining. Primary antibody to cyclin A (dilution 1:200) was applied for 1 hr, at 4°C, and antibody for p16 (dilution 1:300) was applied overnight at 4°C. The streptavidin-biotin method using DAKO's LSAB+ kit (DAKO Cytomation, Glostrup, Denmark) was used, with diaminobenzidine (DAB) as the chromogen solution and Mayer's hematoxylin for the counterstain. Normal lymph node tissue was included in every staining procedure as positive control for cyclin A and p16, whereas incubation with the pure antibody diluent (without the primary antibody) served as a negative control.

Evaluation of immunostaining

Brown-colored products in the nucleus identified positive staining. The results of immunohistochemical staining were scored by quantitative technique: absence of staining in all tumor cells (negative staining-0); positive staining (1-100%; Figures 2,3).
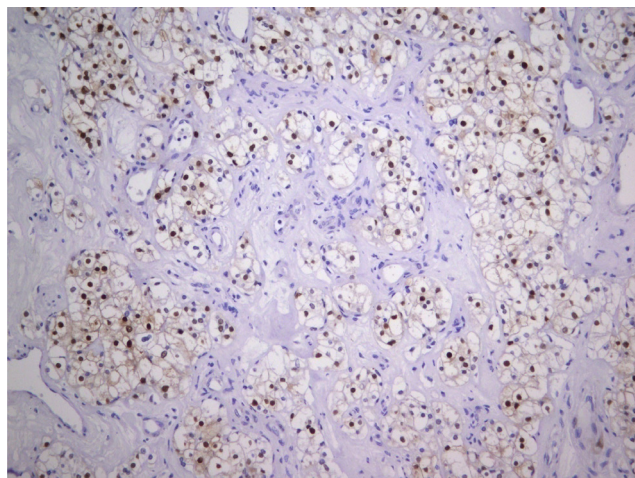
Figure 2. Cyclin A: clear cell type, nuclear grade II, stage II, positive staining 40%, 200x.
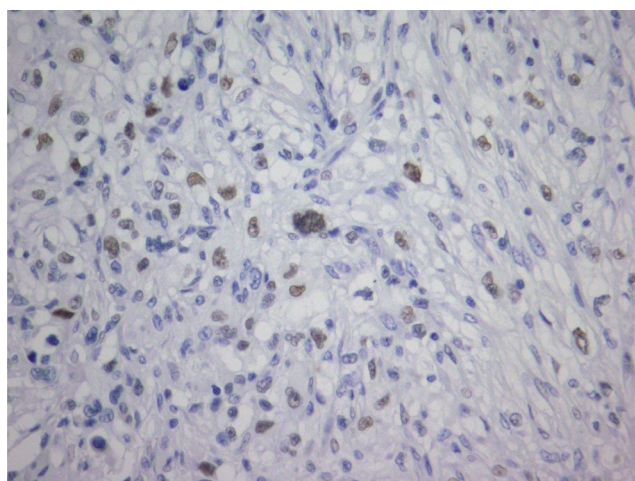

Figure 3. p16: clear cell type, nuclear grade III, stage II, positive staining 30%, 400x.

Statistics

For the analysis of primary data, descriptive statistical methods, statistical hypothesis testing and assessment methods for correlation were used. From descriptive statistical methods used were measure of central tendency (mean), measure of variability (standard deviation) and relative numbers. To test the hypothesis about the difference of frequency Pearson's chi-square test and Fischer's exact test were used. To test the hypothesis about the difference of numerical values the T-test and Mann-Whitney U test were used. For evaluation of correlation between immunohistochemical expression of cyclin A and tumor size we used Spearman's coefficient of correlation. For non-parametric statistical significance test Kruskal-Wallis test was used. The statistical significance was set at $p<0.05$. The median was done when the standard deviation divided by the arithmetic mean was greater than 50%.

Results

In lower stages the median value of cyclin A expression was 7.5% (range 0-70), and of p16 expression was 15% (range 0-80), whereas in higher stages the median value of cyclin A expression was 10% (range 0-60) and of p16 expression was 20% (range 0-75). The difference of expression of cyclin A and p16 in different stages did not show statistical significance (Table 1). In lower grade RCC the median value of cyclin A expression was 5% (range 0-50) and of p16 expression was 15% (range 0-80), whereas in higher grade RCC, the median value of cyclin A expression was 10% (range 0-70) and of p16 expression was 20% (range 0-80). There was no significant

Table 1. Median value for cyclin A and p16 in relation to stage, grade, histological type and deadly outcome in patients with RCC

	Stage	Nuclear grade				
	Stage	Lower	Higher	Total	p	Lower	Higher	Total	p
Cyclin A	Med	7.5	10.0	10.0	0.863	5.0	10.0	10.0	0.291
	Min	0.0	0.0	0.0		0.0	0.0	0.0	
	Max	70.0	60.0	70.0		50.0	70.0	70.0	
p 16	Med	15.0	20.0	20.0	0.913	15.0	20.0	20.0	0.421
	Min	0.00	0.0	0.0		0.0	0.0	0.0	
	Max	80.0	75.0	80.0		80.0	80.0	80.0	

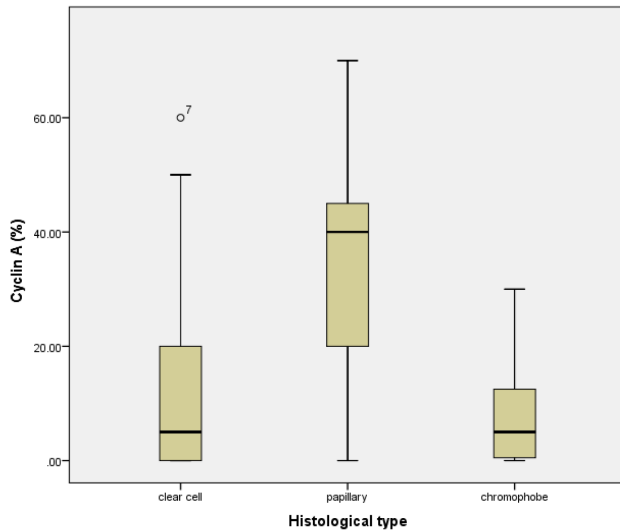| | | \multicolumn{5}{c}{Histological type} | \multicolumn{4}{c}{Deadly outcome – September 2015} |
|---|---|---|---|---|---|---|---|---|---|---|
| | Stage | Clear cell | Papillary | Chromophobe | Total | p | No | Yes | Total | p |
| Cyclin A | Med | 5.0 | 40.0 | 5.0 | 10.0 | <0,001 | 5.0 | 20.0 | 10.0 | 0.019 |
| | Min | 0.0 | 0.0 | 0.0 | 0.0 | | 0.0 | 0.0 | 0.0 | |
| | Max | 60.0 | 70.0 | 30.0 | 70.0 | | 70.0 | 60.0 | 70.0 | |
| p 16 | Med | 10.0 | 57.5 | 10.0 | 20.0 | <0,001 | 15.0 | 20.0 | 20.0 | 0.469 |
| | Min | 0.0 | 35.0 | 0.0 | 0.0 | | 0.0 | 0.0 | 0.0 | |
| | Max | 60.0 | 80.0 | 35.0 | 80.0 | | 80.0 | 70.0 | 80.0 | |

Figure 4. Median value for cyclin A in relation to histological type (p<0.001).
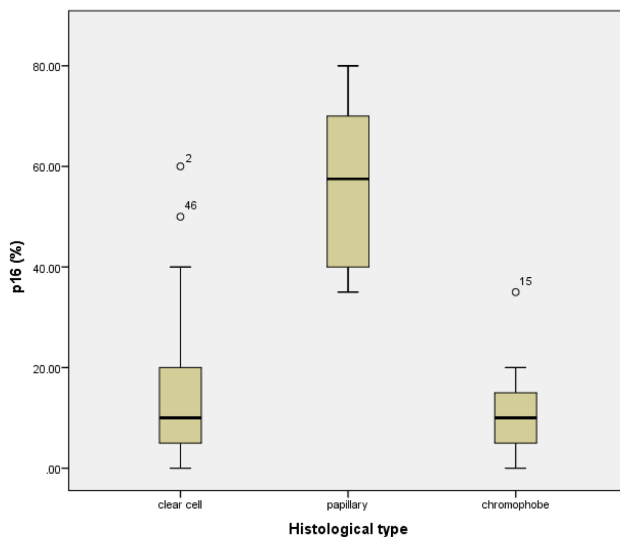

Figure 5. Median value for p16 in relation to histological type (p<0.001).
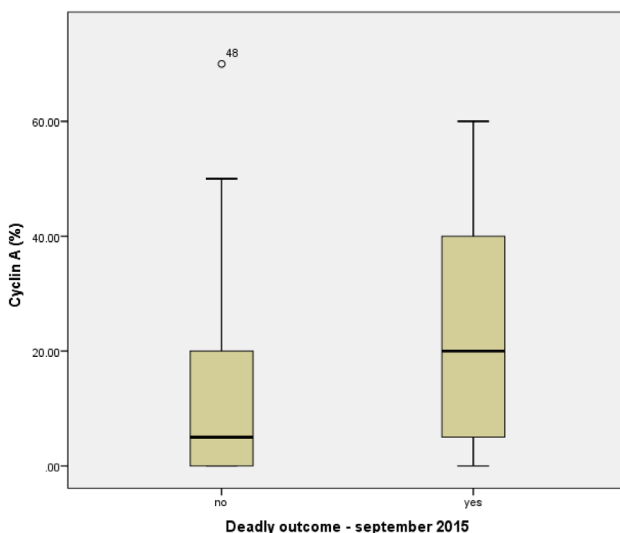

Figure 6. Median value for cyclin A in relation to deadly outcome (September 2015) (p=0.019).

difference of expression of cyclin A and p16 in different grades. The highest median values of cyclin A (40%; range 0-70; Figure 4) and p16 expressions (57.5%; range 35-80; Figure 5) were in the papillary histological type. There was no statistically significant correlation between the expression of cyclin A (ro=0.158; p=0.179; Table 2), expression of p16 (ro=0.122; p=0.302) and size of tumors. Survival analysis showed that in the group of patients that had died before September 2015, the median value for cyclin A was 20% (range 0-60), which was significantly higher than 5% (range 0-70), found in the group of patients that survived (p=0.019; Figure 6). The expression of p16 was similar in both groups (15%; range 0-80) in the group that survived and 20% (range 0-70) in the group of patients who died before September 2015.

Table 2. Median value for cyclin A and p16 in relation to mean tumor size

	Correlations		Tumor size – mean value (mm)
Spearman's rho	Cyclin A (%)	ro	0.158
		p	0.179
		N	74
	p16 (%)	ro	0.122
		p	0.302
		N	74

Discussion

This study included 74 patients, among whom 65% males, the results of which are consistent with data from literature [2]. Analyzing the immunohistochemical expression of cyclin A in relation to stage (median value for lower stage 7.5% and 10% for higher), it was noticed that the expression was higher with increasing stage of disease, however the results did not reach statistical significance. A study which examined the expression of cyclin A in endometrial cancer also showed an increase of the expression in higher stages and more aggressive tumors [15]. According to Jernman et al., high expression of both cyclin A and Ki67 in rectal neuroendocrine tumors was associated with tumor metastasis [16].

Although p16 is thought to be a tumor suppressor protein, we have found that the median value of p16 expression was also higher with higher stage. Contrary to our results, Tanaka et al. showed that inactivation of p15 and p16 correlated with higher grade, invasive and metastatic RCC [17]. This information could direct research

towards finding the factors that influence the biological behavior of p16, and possibly modify its primary role.

Loss of balance between the cyclins, cyclin dependent kinases and their inhibitors, leads cells toward malignant transformation [6]. The expression of cyclin A and p16 was lower in nuclear grade I and II, compared to higher grade, even though the results did not reach statistical significance. The results obtained for cyclin A were expected, considering its role in the cell cycle, however, we expected p16, a tumor suppressor protein, to have a lower expression in higher nuclear grades. A study done by the Ikureowo et al. suggests that absence of p16 expression may indicate RCC tumorigenesis [14]. Our results did not show the expected decrease of p16 expression in higher grades. Another research on thyroid tumors also showed that loss of p16 is not required for malignant transformation, but it can be one of the factors [18]. On the other hand, research of cyclins A and E revealed the important role of increased expression of these cyclins in developing endometrial carcinogenesis in patients with breast cancer [19]. Cyclin A has prominent role in the emergence of non-small cell lung cancer [20] and acute myeloid leukemia [21].

We analyzed the expression of p16 and cyclin A in relation to the size of the tumor, and we observed no statistical difference. The highest expression of cyclin A was 70% (mean tumor size 61.6 mm), and expression of p16 was 80% in two cases (61.6 and 21.6 mm mean tumor size).

An association was noticed between cyclin A expression and survival duration. Subjects with deadly outcome had significantly higher median values of cyclin A expression (median 20%) compared to the group of survivors. Another research on RCC, that analyzed survival without recurrent disease, showed that higher expression of cyclin A was correlated with poor prognosis [12]. Similar to these findings, the research conducted by Mu et al. on breast cancer has shown that high expression of Ki67 and cyclin A unequivocally indicated poor prognosis in breast cancer [22]. All of these results imply that high cyclin A expression indicates unfavorable prognosis and leads to the conclusion that cyclin A could be a potential prognostic marker.

Contrary to the results of cyclin A expression, we did not observe a significant difference in p16 expression among groups with different survival duration. However, in a previous study of p16 expression in RCC cases, high expression of p16 was associated with a significantly better prognosis and longer survival, suggesting a probable role of p16 in the development of RCC [14]. A study by Tanaka et al. showed that inactivation of p15 and p16 is one of the main pathways of oxystress-induced carcinogenesis in RCC in rats [17]. Other authors pointed out the important role of p16 in the process of cellular ageing [23]. Vidauretta et al. showed that hypermethylation of p16 promoter is the most common mechanism for p16 gene silencing, leading to a longer survival rate in patients with RCC [13].

Regarding histological type, we have noticed that the papillary type had the highest median value of both cyclin A expression (40%; range 0-70) and p16 expression (57.5%; range 35-80). We also noticed that the expression of cyclin A and p16 was significantly higher in the papillary type compared to other types of RCC, which stresses the potential of these two molecules as diagnostic markers in this histological type. The expression of p16 in different histological types of tumors of the same location was assessed in a study of lung tumors where neuroexocrine lung tumors had low expression of p16, contrary to neuroendocrine lung tumors [24].

These results may encourage research on the interactions between cyclin A and p16 as proteins with antagonistic effects. More detailed analysis of increased expression of the proto-oncogene and tumor suppressor gene at the same time in specific histological types of tumors could point out a molecular mechanism that enables the dominance of one of them in cancer development.

Aknowledgements

Conflict of interests

The authors declare no conflict of interests.

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