CANCER UROLOGY 3'2016 VOL. 12

Prognostic value of the expression of carbonic anhydrase 9 in combination with other markers in patients with clear cell renal cell carcinoma

N.A. Gorban'[1], A.M. Popov[2], O.B. Karyakin[2]

[1]Federal State-Owned and State-Financed Enterprise "Chief Military Clinical Hospital named after academician N.N. Burdenko"; 3 Gospitalnaya place, Moscow, 105094, Russia;
[2]Federal State Budgetary Institution "National Medical Research Radiological Center" of the Russian Ministry of Health; 4 Koroleva St., Obninsk, Kaluga Region, 249036, Russia

Background. *The proportion of renal cell carcinoma (RCC) in the structure of oncological incidence in Russia is 3.9 %. This nosology has a leading position by the growth rate. The number of new cases of RCC from 2004 to 2014 increased by 42.9 %. Carbonic anhydrase (CA) enzymes are transmembrane enzymes that play an important role in pH regulation catalyzing reversible reactions of carbonic acid to carbon dioxide and water. Recently we have seen studies on prognostic and predictive value of CA9 expression in clear cell RCC.*
Objective *– reveal relationship between CA9 expression and proliferative activity, apoptosis, morphological picture and clinical course of a tumor.*
Materials and methods. *The study included 67 patients (47 men and 20 women) aged from 32 to 73 years (55.0 ± 7.6 years), suffering from clear cell RCC. All the patients were treated at the Medical Radiological Research Center. Follow-up period lasted from 8 to 116 months (mean – 36.5 months). Patients underwent nephrectomy, histological study with Fuhrman nuclear grading, immunohistochemistry with antibodies against p53, bcl-2, Ki-67 and CA9.*
Results and conclusions. *CA9 expression is associated with the expression of bcl-2, while the lack of CA9 expression is associated with p53. Loss of CA9 expression is a poor prognostic factor and it is associated with the development of metastasis and recurrence of the disease, as well as lower disease-free survival.*

Key words: *carbonic anhydrase, clear cell renal cell carcinoma, proliferative activity, apoptosis, prognostic factors, survival*

DOI: 10.17650/1726-9776-2016-12-3-40-44

Introduction

In Russia, renal cell carcinoma (RCC) comprises 3.9 % of all cancers in the structure of oncological morbidity. Its rate of increase is one of the largest among all nosologies. In the period from 2004 to 2014, the number of new RCC cases increased by 42.9 % [1]. Currently, there aren't any prognostic markers for RCC [2, 3]. Carbohydrase 9 (CA9) is expressed in various histological subtypes of RCC. The majority (95 %) of clear cell tumors are characterized by high and homogenous CA9 expression; in oncocytomas, chromophobe, and papillary renal cell carcinomas its expression is significantly lower [4].

Carbohydrase 9 (G250) was first described in 1986 in a study of the G250 monoclonal antibody tumor specificity [5]. CA9 is a transmembrane glycoprotein of the carbohydrase enzyme family. These enzymes play a crucial role in regulation of excessive proton generation in the cell and, therefore, pH control [6, 7]. Expression of CA9 is observed in almost all types of tumors (cancer of the uterine cervix, esophagus, lung, breast, brain, vulva where expression is heterogenous) and is associated with hypoxia [8].

Lately, several studies were published considering prognostic and predictive significance of CA9 in clear cell RCC [9–12]. Prognostic markers in clear cell RCC are Ki-67 reflecting proliferative level of the tumor and apoptotic markers p53 and bcl-2 [13].

The study objective is to investigate a relationship between CA9 and tumor proliferative activity, apoptosis, morphological characteristics, and clinical picture.

Materials and methods

The study included 67 patients (47 men, 20 women) aged 32–73 years (55.0 ± 7.6 years) with clear cell RCC. Patients were treated at the Medical Research Radiological Center. Follow-up duration varied from 8 to 116 months (mean 36.5 months). All patient underwent nephrectomy. After surgery, patients with advanced cancer were prescribed 1st line targeted therapy in accordance with the guidelines of the European Association of Urology (EAU).

Histological examination was performed using a large number of tumor fragments (considering its heterogeneity) per the developed protocol. Histological sections of tumor tissue were stained with hematoxylin and eosin and subsequently used for immunohistological examination. Endogenous peroxidase was blocked with cooled 3 % hydrogen peroxide solution for 10 minutes. In order to restore the antigen structure of paraffin-embedded material fixed with formaldehyde, histological sections were warmed in a water bath with 0.01 M citrate buffer (pH 6.0) for 20 minutes. Incubation with primary antibodies (Dako) was performed at room temperature for 60 minutes. Streptavidin-biotin-

peroxidase method was used for visualization of immune reaction products (EnVision+ System-HRP, Dako), diaminobenzidine solution was used as chromogenic substrate (Liquid DAB+, Dako), nuclei were stained by hematoxylin. Sections with applied secondary antibodies, without primary antibodies, were used as control.

Grade of tumor differentiation was evaluated per Fuhrman in accordance with the 2004 guidelines of the World Health Organization [14]. Brown staining of tumor cell nuclei and p53-specific nuclear staining in more than 10 % of tumor cells indicated positive expression of the Ki-67 and p53 proteins, respectively. Positive expression of bcl-2 was characterized by specific cytoplasmic staining of more than 75 % of tumor cells, positive expression of CA9 was characterized by specific membrane staining of more than 75 % of tumor cells.

Morphological examination of tumors was performed using the OpenCVTool software for image processing, digital scanner MIRAX MIDI (Zeiss), Intel Core I3 CPU with 4 Gb RAM.

Ki-67 index was evaluated based on analysis of more than 1,000 cells. Ki-67 index was calculated as percentage of specifically stained nuclei among all nuclei.

Survival was analyzed using the Kaplan-Meier estimator (1958), significance of differences between survival curves was confirmed using the Cox's F-Test. Level of significance was $p \leq 0.05$. Statistical data processing of the obtained parameters was performed using the Statistica 10.0 software package.

Results

At the time of diagnosis, 31 (46.3 %) patients had localized cancer and 36 (53.7 %) patients had metastatic cancer. During the follow up, in 13 patients with localized tumor at time of diagnosis progression was observed at various times after surgical treatment.

In all cases, clear cell RCC was morphologically confirmed with the following distribution by the Fuhrman's grade: grade 1–3, grade 2–17, grade 3–23, and grade 4–24 patients. In 13 (19.4 %) tumors a sarcomatoid component was identified. Necrotic lesions were reported in 35 cases (52.2 %).

We denoted 31 tumors (46.3 %) as CA9-negative. Only in 10 (14.9 %) of them expression was entirely absent, and in 21 expression was weak and localized.

Mean level of Ki-67 was 9.7 ± 1.3 % (from 0.1 to 49.0 %); Ki-67 above 10 % was identified in 22 patients.

Nuclear expression of p53 was observed in 19 (28.4 %) patients. It varied both by number of stained cells and intensity of staining.

Bcl-2 expression was fully absent in 31 patients, in 9 patients it was localized (these patients were assigned to the bcl-2-negative group), and pronounced cytoplasmic expression was observed only in 27 patients (40.3 %).

In 6 (46 %) cases, the sarcomatoid component expressed p53, and in 100 % of cases it lacked bcl-2 and CA9 expression, while Ki-67 level was above 10 %.

We analyzed CA9 expression and its correlation with clinical and morphological data, as well as other markers. In the group of highly differentiated tumors (grades 1 and 2 per Fuhrman) only 4 of 20 tumors were considered CA9-negative, and even though CA9 expression was localized and weak, it wasn't fully absent (see Table). At grades 3 and 4, 27 of 47 cases were negative (in 10 cases expression was fully absent, in others localized weak staining was observed) ($p = 0.0047$). However, there was no significant correlation with the presence of the sarcomatoid component (see Table) or Ki-67 level.

CA9 expression is associated with decent relapse-free survival ($p = 0.02$) (Fig. 1), and there's a trend for disease-specific survival rate dependence on CA9 expression ($p = 0.098$) (Fig. 2).

Tumor metastatic potential is associated with the loss of CA9 expression ($p = 0.009$) (see Table). Thus, in patients with CA9-expressing tumor metastases were diagnosed in 22 (61.1 %) cases, while in patients lacking CA9 expression – in 27 (87.1 %) cases. Moreover, metastases were observed in 21 of 31 patients with lack of expression at the time of diagnosis, and in 15 of 36 patients in the CA9-positive group ($p = 0.029$).

Expression of p53 is accompanied by loss of CA9 expression ($p = 0.005$), loss of bcl-2 expression is also accompanied by loss of CA9 expression (see Table).

Discussion

Carbonic anhydrases are transmembrane enzymes playing an important role in pH regulation: They catalyze a reversible reaction of carbonic acid conversion into carbon dioxide and water. The majority of CA9 studies consider its expression in different RCC variants [4, 15]. We evaluated prognostic significance of CA9 in clear cell RCC. In our study, a connection between CA9 expression and Fuhrman's differentiation grade is evident. A decrease in the differentiation grade is associated with decreased CA9 expression or its loss. Similar results were obtained by other researchers [8, 15, 16]. We haven't found a correlation with the presence of sarcomatoid component. However, it should be noted that in our study CA9 expression was never observed in the sarcomatoid component, but in more differentiated areas expression could be high, and these tumors were considered CA9-positive. Presumably, evaluation of CA9 expression should take into consideration RCC heterogeneity, as well as consider the presence of sarcomatoid component an unfavorable factor irrespective of its volume. The presence of sarcomatoid component reflects tumor dedifferentiation accompanied by loss of its morphological and immunohistochemical characteristics. The sarcomatoid component has a high level of proliferative activity reflected by Ki-67, frequent expression of the p53 oncoprotein, and loss of bcl-2 expression, and therefore the loss of CA9 expression is natural.

In our previous study [13], Ki-67 was associated with high nuclear grade and poor prognosis of clear cell RCC, and its level above 10 % – with metastasis and low metastasis-free survival. Decrease in differentiation is associated with increased proliferative activity, and a correlation between CA9 expression and Ki-67 level should be expected, but here there wasn't any significant correlation. Supposedly, these are independent prognostic factors.

In many studies [17, 18] including ours [13], p53 expression is an unfavorable prognostic factor in RCC associated with disease progression, metastasis, and low cancer-specific survival. Our data show that in tumors with *p53* gene mutation and expression of the p53 protein, CA9 expression is lower or absent, though lack of p53 expression does not guarantee preservation of CA9. There's a direct connection between bcl-2 and CA9 expression: The majority of tumors expressing bcl-2 express CA9, and loss of bcl-2 means loss of CA9.

Loss of CA9 expression is associated with such unfavorable prognostic factors as p53 expression and loss of bcl-2 expression. Lack of CA9 expression is an unfavorable prognostic factor for clear cell RCC associated with metastasis, and in most cases the disease is already metastatic at the time of diagnosis.

The connection between loss of CA9 expression and metastases was also demonstrated by other researchers [19]. However, M.H. Bui et al. [20] have shown that low CA9 expression in primary clear cell RCC was an unfavorable predictor of survival in a study including 321 patients. Furthermore, according to their data, in patients with localized cancer CA9 doesn't have prognostic significance (no difference in survival), but in metastatic cancer low expression level reflects median cancer-specific survival: 5.5 months vs. 24.8 months with high expression level. Moreover, Z. Pan et al. have shown that CA9 expression in metastases is even higher than in the primary tumor, but they didn't propose an explanation for this phenomenon [21].

Our results show that CA9 expression is associated with high relapse-free survival ($p = 0.02$), though dependence of cancer-specific survival on CA9 expression can only be considered a tendency ($p = 0.098$). Our data agree with a literature meta-analysis performed by Z. Zhao et al. to refine the prognostic role of CA9 in RCC [22]. According to their conclusions, multiple studies evaluating CA9 as

Relationships between CA9 expression, clinical symptoms, and expression of other markers

Characteristic	CA9+	CA9–	*p*
Grades 1 and 2	16 (23.9 %)	4 (6 %)	0.0047
Grades 3 and 4	20 (29.8 %)	27 (40.3 %)	
Presence of the sarcomatoid component	5 (7.5 %)	8 (11.9 %)	0.18
Absence of the sarcomatoid component	31 (46.3 %)	23 (34.3 %)	
Localized cancer	22 (32.8 %)	27 (40.3 %)	0.009
Metastatic cancer	14 (20.9 %)	4 (6.0 %)	
Ki-67 > 10 %	9 (13.4 %)	13 (19.4 %)	0.11
Ki-67 < 10%	27 (40.3 %)	18 (26.9 %)	
P53+	5 (7.5 %)	14 (20.9 %)	0.005
P53–	31 (46.3 %)	17 (25.4 %)	
bcl-2+	19 (28.4 %)	8 (11.9 %)	0.02
bcl-2–	17 (25.4 %)	23 (34.3 %)	

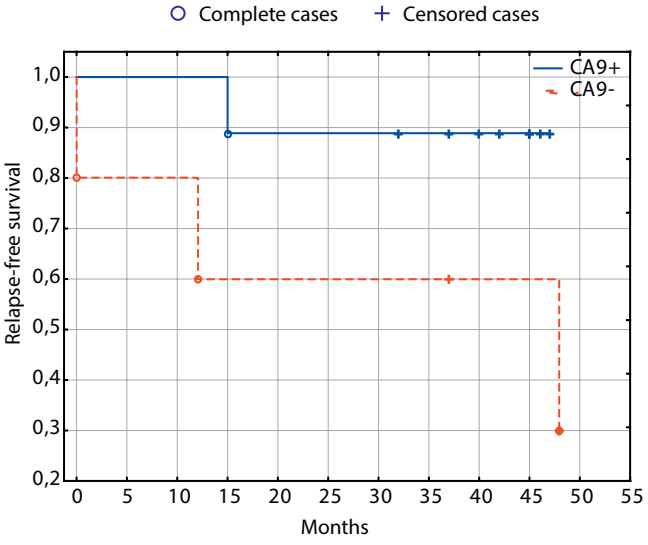

Fig. 1. *Dependence of relapse-free survival on CA9 expression*

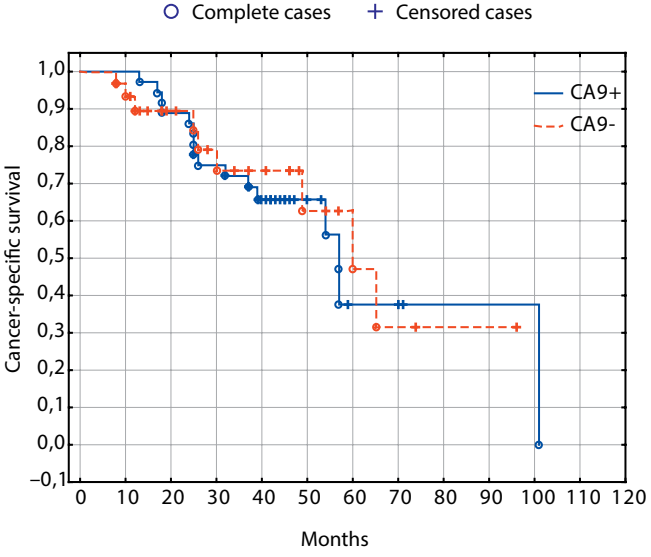

Fig. 2. *Dependence of cancer-specific survival on CA9 expression*

a prognostic marker for RCC obtained contradictory results, but most data allow to assume that low CA9 expression is associated with low cancer-specific survival ($p = 0.006$), poor overall survival ($p = 0.002$), and progression-free survival ($p = 0.02$). Some studies haven't found any prognostic significance of CA9 expression [8, 23].

Our results and literature data allow to consider CA9 a valuable prognostic biological marker for clear cell RCC progression. Moreover, lately CA9 was put forward as a potential effective biological marker for selection of optimal treatment in individual patients.

Conclusion

Loss of CA9 expression is an unfavorable prognostic factor for clear cell RCC associated with metastasis and disease recurrence, low relapse-free survival.

REFERENCES

1. Malignant tumors in Russia in 2014 (morbidity and fatality). Ed. by: A.D. Kaprin, V.V. Starinskiy, G.V. Petrova. Moscow, 2016.
2. Matveev V.B., Volkova M.I. Kidney cancer. Russkiy meditsinskiy zhurnal = Russian Medical Journal 2007;15(14):1094.
3. Alekseev B.Ya., Kalpinskiy A.S., Nyushko K.M. et al. Determination of factors for the forecast of the efficiency of therapy with bevacizubam at patients with metastatic renal cell carcinoma. Onkourologiya = Oncourology 2013;(3):17–23.
4. Takacova M., Bartosova M., Skvarkova L. et al. Carbonic anhydrase IX is a clinically significant tissue and serum biomarker associated with renal cell carcinoma. Oncol Lett 2013;5(1):191–7. PMID: 23255918; PMCID: PMC3525455; DOI: 10.3892/ol.2012.1001.
5. Oosterwijk E., Ruiter D.J., Hoedemaeker P.J. Monoclonal antibody G250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. Int J Cancer 1986;38(4):489–94. PMID: 2428759.
6. Oosterwijk-Wakka J.C., Boerman O.C., Mulders P.F., Oosterwijk E. Application of Monoclonal Antibody G250 Recognizing Carbonic Anhydrase IX in Renal Cell Carcinoma. Int J Mol Sci 2013;14(6): 11402–23. PMID: 23759990; PMCID: PMC3709739; DOI: 10.3390/ijms140611402.
7. Stillebroer A.B., Mulders P.F., Boerman O.C. et al. Carbonic anhydrase IX in renal cell carcinoma: implications for prognosis, diagnosis, and therapy. Eur Urol 2010;58(1):75–83. PMID: 20359812.
8. Leibovich B.C., Sheinin Y., Lohse C.M. et al. Carbonic anhydrase IX is not an independent predictor of outcome for patients with clear cell renal cell carcinoma. J Clin Oncol 2007;25(30):4757–64. PMID: 17947723; DOI: 10.1200/JCO.2007.12.1087.
9. Muriel López C., Esteban E., Berros J.P. et al. Prognostic factors in patients with advanced renal cell carcinoma. Clin Genitourin Cancer 2012;10(4): 262–70. DOI: 10.1016/j.clgc.2012.06.005.
10. Muriel López C., Esteban E., Astudillo A. et al. Predictive factors for response to treatment in patients with advanced renal cell carcinoma. Invest New Drugs 2012;30(6): 2443–9. DOI: 10.1007/s10637-012-9836-4.
11. Zhang B.Y., Thompson R.H., Lohse C.M. et al. Carbonic anhydrase IX (CAIX) is not an independent predictor of outcome in patients with clear cell renal cell carcinoma (ccRCC) after long-term follow-up. BJU Int 2013;111(7):1046–53. DOI: 10.1111/bju.12075.
12. Tostain J., Li G., Gentil-Perret A., Gigante M. Carbonic anhydrase 9 in clear cell renal cell carcinoma: a marker for diagnosis, prognosis and treatment. Eur J Cancer 2010;46(18):3141–8. DOI: 10.1016/j.ejca.2010.07.020.
13. Gorban' N.A., Ivanova S.V., Karyakin O.B. et al. Clinical value of both proliferation markers and apoptosis in the clear-cell carcinoma. Onkourologiya = Oncourology 2014;(1):30–5.
14. World Health Organization Classification of Tumours. Pathology & Genetics: Tumours of the Urinary System and Male Genital Organs. Lyon, France: IARC Press, 2004.
15. Genege E.M., Ghebremichael M., Najarian R. et al. Carbonic anhydrase IX expression in renal neoplasms: correlation with tumor type and grade. Am J Clin Pathol 2010;134(6):873–9. PMID: 21088149; PMCID: PMC3778911; DOI: 10.1309/AJCPPPR57HNJMSLZ.
16. Mickley A., Kovaleva O., Kzhyshkowska J., Gratchev A. Molecular and immunologic markers of kidney cancer-potential applications in predictive, preventive and personalized medicine. EPMA J 2015;6:20. DOI: 10.1186/s13167-015-0042-2.
17. Uzunlar A.K., Sahin H., Yilmaz F., Ozekinci S. Expression of p53 oncoprotein and bcl-2 in renal cell carcinoma. Saudi Med J 2005;26 (1):37–41. PMID: 15756350.
18. Warburton H.E., Brady M., Vlatković N. et al. p53 regulation and function in renal cell carcinoma. Cancer Res 2005;65(15):6498–503. PMID: 16061625; DOI: 10.1158/0008-5472.CAN-05-0017.
19. Chamie K., Klöpfer P., Bevan P. et al. Carbonic anhydrase-IX score is a novel biomarker that predicts recurrence and survival for high-risk, nonmetastatic renal cell carcinoma: Data from the phase III ARISER clinical trial. Urol Oncol 2015;33(5):204. e25–33. PMID: 25823535; DOI: 10.1016/j.urolonc.2015.02.013.
20. Bui M.H., Visapaa H., Seligson D. et al. Prognostic value of carbonic anhydrase IX and KI67 as predictors of survival for renal clear cell carcinoma. J Urol 2004;171(6 Pt 1): 2461–6. PMID: 15126876.
21. Pan Z., Grizzle W., Hameed O. Significant variation of immunohistochemical marker expression in paired primary and metastatic clear cell renal cell carcinomas. Am J Clin Pathol 2013;140(3):410–8. PMID: 23955461; DOI:10.1309/AJCP8DMPEIMVH6YP.
22. Zhao Z., Liao G., Li Y. et al. Prognostic value of carbonic anhydrase IX immunohistochemical expression in renal cell carcinoma: a meta-analysis of the literature. PLoS One 2014;9(11):e114096. PMID: 25426861; PMCID: PMC4245260; DOI: 10.1371/journal.pone.0114096.
23. Choueiri T.K., Cheng S., Qu A.Q. et al. Carbonic anhydrase IX as a potential biomarker of efficacy in metastatic clear-cell renal cell carcinoma patients receiving sorafenib or placebo: analysis from the treatment approaches in renal cancer global evaluation trial (TARGET). Urol Oncol 2013;31(8):1788–93. DOI: 10.1016/j.urolonc.2012.07.004.