Prognostic Significance of CD44 Molecule in Renal Cell Carcinoma

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Aim. To analyze the expression and the prognostic value of CD44s and variant v5 and v6 isoforms in a large series of conventional renal cell carcinomas.

Methods. The expression of CD44 isoforms was immunohistochemically evaluated in 173 conventional renal cell carcinomas, and was compared with the usual clinicopathological parameters such as tumor size, histological grade, pathological stage, and Ki-67 proliferative index. The relationship of the CD44 expression to the cancer-specific survival was evaluated by log rank test.

Results. While normal renal tissue was negative for CD44s protein, it was upregulated in 70 (40.5%) out of 173 carcinomas, and its expression significantly correlated with histological grade (p<0.001), pathological stage (p=0.023), and Ki-67 proliferative index (p<0.001). Moreover, the expression of CD44s protein was an adverse prognostic parameter in univariate survival analysis for patients with tumors expressing high levels of CD44s protein (p=0.003). CD44v5 and v6 isoforms were expressed in 11 (6.4%) and 28 (16.2%) tumors, respectively. Their expression was significantly higher in tumors with higher histological grade (p=0.001 and p=0.001, respectively). Also, the expression of v6 isoform was higher in tumors with high proliferation activity (p=0.001).

Conclusion. CD44s molecule may play a role in the progression of conventional renal cell carcinoma, and may be used in the evaluation of disease outcome.

Key words: adenocarcinoma; carcinoma, renal cell; CD44S antigen; CD44v5 antigen; CD44v6 antigen; cell adhesion; immunohistochemistry; prognosis

Renal cell carcinoma is a common cancer in Western countries, with increasing incidence and extremely variable clinical course (1). At present, tumor stage and nuclear grade are considered to be the main prognostic indicators (2). However, in the significant number of patients, these parameters are insufficient to predict biological behavior of the tumor, especially in conventional renal cell carcinomas, the most common type of renal cell carcinoma (3). Therefore, additional prognostic factors are needed to identify patients at high risk of tumor progression.

Adhesion molecules involved in cell-cell and cell-matrix interactions play a key role in the process of tumor development and the metastatic cascade (4). Among them, CD44 molecule possesses a number of properties that are required by a metastasizing cell (5). It has been shown to confer metastatic potential in rat pancreatic carcinoma (6). CD44 is a ubiquitous cell surface molecule involved in the aggregation, migration, and activation of cell and presentation of growth factors. It has been shown to display a great molecular heterogeneity owing both to alternative exon use and variegated posttranslational modifications. The CD44 gene consists of 20 exons, only 10 of which are normally expressed, encoding the hematopoietic or standard form of CD44 (CD44s). Additional 10 exons, encoding extracellular regions, are expressed by alternative splicing of the nuclear RNA, generating a large number of variant isoforms (CD44v).

The expression of CD44 and its variants has been correlated with clinical outcome in several human malignancies, although these findings are not consistent among the studies (7-10).

The aim of our study was to analyze the expression of CD44s molecule in conventional renal cell carcinoma and assess the relationship between CD44 expression, clinicopathologic variables, and patient survival. In addition, the expression of CD44v5 and v6 splice variants was also evaluated for their relation to clinicopathological prognostic parameters.

Patients and Methods

Case Selection

All the consecutive cases of conventional renal cell carcinoma which could be retrieved from the computer files of the Department of Pathology, Rijeka University School of Medicine, and for which the 5-year survival data could be obtained, were included in the study. This corresponded to 213 cases diagnosed between 1990 and 1998. Since typing and grading of renal cell carcinoma have markedly changed in the past decade, all hematoxylin-eosin stained sections from individual case were re-
viewed by 2 pathologists. This excluded 23 cases which did not meet the criteria for conventional renal cell carcinoma diagnosis according to Heidelberg classification (11). Additional 17 cases were excluded on the basis of incomplete clinical data or inadequate archival material, so a total of 173 specimens were finally included in the study. Further 57 patients were lost during the follow-up, so a total of 116 cases were included in the survival analysis (Fig. 1).

Table 1. Clinicopathological characteristics of patients and tumors

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)*</td>
<td>104/69</td>
</tr>
<tr>
<td>Nuclear grade:†</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>30</td>
</tr>
<tr>
<td>G2</td>
<td>69</td>
</tr>
<tr>
<td>G3</td>
<td>45</td>
</tr>
<tr>
<td>G4</td>
<td>29</td>
</tr>
<tr>
<td>Pathological stage:*</td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>78</td>
</tr>
<tr>
<td>pT2</td>
<td>42</td>
</tr>
<tr>
<td>pT3</td>
<td>53</td>
</tr>
<tr>
<td>pT4</td>
<td>0</td>
</tr>
<tr>
<td>Tumor size (cm):</td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>45</td>
</tr>
<tr>
<td>4-7</td>
<td>48</td>
</tr>
<tr>
<td>&gt;7</td>
<td>80</td>
</tr>
</tbody>
</table>

* M/F, male/female.
† Fuhrman’s nuclear grade (2).
‡ TNM classification of the International Union Against Cancer (12).

Figure 1. Scheme of the study. CRCC – conventional renal cell carcinoma.

Clinicopathological Data
Clinicopathological data were obtained from patients’ medical records at the Surgical Department, Rijeka University Hospital Center, and from pathologic reports. Clinicopathological features of tumors are summarized in Table 1. There were 104 men and 69 women in the study. The mean size of tumors was 7.2 ± 3.5 cm. Tumors were graded using the Fuhrman’s nuclear grading system (2), and the grading distribution was as follows: 30 (17.3%) grade 1, 69 (39.9%) grade 2, 45 (26%) grade 3, and 29 (16.8%) grade 4 tumors. Tumor stage was defined according to the International Union Against Cancer (UICC) 1977 tumor-node-metastasis (TNM) classification (12). There were 120 (69.4%) tumors limited to the kidney (pT1 and pT2) and 53 (30.6%) tumors that expanded outside the kidney (pT3 and pT4). At the time of surgery, 12 patients had distant metastases. Follow-up information was obtained from patients’ medical records and from files of the Croatian Cancer Registry. The follow-up was available for 116 patients and ranged from 1 to 165 months (median 85 months). Survival time was calculated from the date of surgery to the date of death or to the date of the last follow up.

Immunohistochemistry
For each case of renal cell carcinoma, a representative slide of the tumor with the highest nuclear grade, and the corresponding paraffin block, was selected. Five-micron sections were cut on glass slides and air-dried over the night. Following deparaffinization in xylene and rehydration in alcohols, heat-induced epitope retrieval was achieved by immersing slides in 10 mmol/L citrate buffer (pH 6.0) and boiling for 10 min in a pressure cooker. Slides were allowed to cool down for 45 minutes. Non-specific antibody blocking was blocked with phosphate-buffered saline (PBS) containing normal goat serum (DAKO, Glostrup, Denmark) for 30 minutes. The sections were then blocked with 5% non-fat dry milk at +4°C with primary antibodies and then proceeded to indirect immunoperoxidase staining in automated immunostainer (DAKO, TechMate, Glostrup, Denmark). All monoclonal antibodies against CD44 molecule were of mouse origin and purchased from Bender MedSystems (Vienna, Austria): anti-CD44s (clone SFF304, dilution 1:15,000), anti-CD44v5 (clone VFF-8, dilution 1:20,000), and anti-CD44v6 (clone VFF-18, dilution 1:15,000). For the negative control, an irrelevant mouse monoclonal IgG antibody was used (DAKO). For positive controls, a staining of intratumoral lymphocytes for CD44s, and urothelium for variant isoforms, was used. In some doubtful cases, a staining with anti-CD68 (clone KP-1, DAKO, dilution 1:200) was performed to distinguish between tumor cells and histiocytes, which are also CD44 positive. The proliferative activity was assessed by detecting the Ki-67 protein with the monoclonal antibody (clone MIB-1, DAKO, dilution 1:50).

Evaluation of Staining
Immunohistochemical staining results were read separately by 2 pathologists, who were blinded to the grade or other clinical parameters of an individual case. Immunostaining of tumor cells was semiquantitatively scored according to four grades as follows: no positive cells (grade 0); fewer than 25% of reactive cells (grade 1), between 25% and 75% of positive cells (grade 2); and more than 75% of cytoplasmic staining (grade 3). Ki-67 labeling index was determined by scoring 500 tumor cells at 400× magnification in tumor areas with the highest density of positive cells. The counting was performed with the Issa 3.1 software (Vams, Zagreb, Croatia). Staining was considered positive if any nuclear staining was seen.

Statistical Analysis
The association of immunohistochemical staining for CD44s, and its v5, and v6 variants with Fuhrman nuclear grade, tumor size and pathological stage was assessed using chi-square test. For the purpose of statistics, all positive cases were grouped together. The relationship with Ki-67 proliferative index, which represents continuous variable, was assessed using Student’s t-test. The association of immunohistochemical staining for CD44s with patient survival was evaluated using the Kaplan-Meier method, and differences between groups were tested by the log rank test. Cox regression analysis was used to perform multivariate statistical analysis. Statistical differences with p value <0.05 were considered significant. Data were analyzed by Statistica 6.1 software (StatSoft, Inc., Tulsa, OK, USA).

Results
Immunohistochemical Expression of CD44 and Variant Isoforms
In the normal renal tissue, no staining for CD44s or variant isoforms was observed, except for a few CD44s- and v5/6-positive tubules, usually in the close vicinity of the tumor tissue (Fig. 2A). Renal cell carcinomas showed heterogeneous staining pattern. It was generally of membranous type, whereas in a few tumors a strong cytoplasmic, dot-like positivity in the paranuclear region was also seen (Fig. 2B). There were 103 (59.5%) CD44s-negative (Fig. 2C), and 70 (40.5%) positive cases (Fig. 2D). Among positive tu-
tumors, 39 (22.5%) tumors were semiquantitatively scored as grade 1 (Fig. 2D), 16 (9.2%) as grade 2, and 15 (8.7%) as grade 3 (Fig. 2E). CD44v5 and v6 isoforms were expressed in only 11 (6.4%) and 28 (16.2%) tumors, respectively. The proliferative activity of tumor cells measured as Ki-67 labeling index had a median value of 4% (range 0.5% to 36.3%).

**Correlations between CD44 Expression and Clinicopathological Characteristics**

The expression of CD44s and variant isoforms was compared with the usual clinicopathological factors, including tumor size, Fuhrman’s nuclear grade, pathological stage, and Ki-67 proliferation index (Table 2). There was no statistically significant difference in CD44s expression in relation to the tumor size (p = 0.556). Regarding the relationship to nuclear grade, only 2 tumors with nuclear grade 1 morphology were positive for CD44s, whereas the positivity increased during progression from grade 1 to grade 4, and this was statistically significant (p < 0.001) (Fig. 3). Considering tumors confined within (ie, pT1 or pT2) and those invading beyond the kidneys (ie, pT3 or pT4) as low-stage and high-stage tumors, respectively, the incidence of predominant expression of CD44s in high pathological stage was significantly higher than that in low-stage tumors (p = 0.023). We also found strong statistical correlation between

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**Figure 2.** Immunohistochemical expression of CD44 isoforms in patients with conventional renal-cell carcinoma. Normal renal tissue is negative for CD44s, except for a few distal tubules (A); membranous and perinuclear dot-like pattern of CD44s expression in conventional renal-cell carcinoma (B); low grade tumor negative (C) or focally positive (D) for CD44s protein, with positive stromal mononuclear cells; high grade tumor strongly and diffusely positive for CD44s (E) and CD44v6 protein (F).
CD44s expression and tumor growth fraction expressed as Ki-67 proliferation index (p<0.001). Namely, CD44s expression significantly increased with the increasing proliferative activity of tumor cells. The mean value of Ki-67 index in CD44s negative tumors was 4.2\%/ 3.8%, whereas it was significantly higher in the group of CD44 positive tumors and reached 9.8\%/ 8.9%. Regarding the expression of v5 and v6 variant isoforms, we found significant association with histological grade (p=0.001, and p=0.001, respectively). Also, the expression of v6 isoform was higher in tumors with high proliferative activity (p=0.001).

Survival Analysis

A significant difference in survival rates was found between patients in relation to different CD44s expression on tumor cells (p=0.003) (Fig. 4). The 5-year survival rate was 68% in patients with CD44s-negative tumors. In patients with CD44s-positive tumors, the 5-year survival rate was 58%, 53%, and 11% for different levels of CD44s positivity (1, 2, and 3, respectively). Since tumor grading and staging are considered major prognostic parameters in CRCC, we first analyzed their impact on postoperative survival. We found a significant inverse correlation between survival and tumor grading (p=0.001) or staging (p=0.001). Although univariate survival analysis showed pathological stage, nuclear grade, and CD44s expression to be significant predictive factors, only nuclear grade (p=0.007) and pathological stage (p=0.006) remained significant in multivariate analysis, whereas CD44s expression did not retain its independent prognostic value (p=0.248) (Table 3). The number of CD44v5/6 positive cases was too small to be included in the survival analysis.

**Table 2. Relationship between CD44s expression and tumor size, grade, stage, and proliferation index**

| Characteristic | CD44 expression (No., %) | | |
|---------------|--------------------------|---|---|---|
| | negative (103; 59.5) | positive (70; 40.5) | p |
| Tumor size (cm, mean±SD): | 7.2 ±3.4 | 6.9 ±3.5 | 0.556 |
| TNM (No.,%):* | pT1,2 | 79 (65.8) | 41 (34.2) | 0.023 |
| | pT3,4 | 25 (47.2) | 28 (52.8) | |
| Fuhrman’s nuclear grade (No.,%): | 1 | 28 (93.3) | 2 (6.7) | <0.001 |
| | 2 | 48 (69.6) | 21 (30.4) | |
| | 3 | 18 (40) | 27 (60) | |
| | 4 | 9 (31) | 20 (69) | |
| Ki-67 index (mean±SD): | 4.2 ±3.8 | 9.8 ±8.9 | <0.001 |

*TNM classification of the International Union Against Cancer (12).
†Student t-test.
‡Pearson’s chi-square test.

**Table 3. Multivariate analysis of prognostic factors in conventional renal cell carcinoma patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk (95% confidence interval)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>CD44s positive</td>
<td>3.25 (0.93-11.35)</td>
<td>0.248</td>
</tr>
<tr>
<td>Higher pT</td>
<td>2.05 (5.10-11.82)</td>
<td>0.006</td>
</tr>
<tr>
<td>Higher nuclear grade</td>
<td>1.7 (3.82-7.85)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**Figure 3.** Expression of CD44s in relation to Fuhrman’s nuclear grade. White bars represent CD44s-negative, and black bars represent positive CD44s-cases. Proportion of positive cases is higher in tumors with higher nuclear grade, p<0.001 (Pearson’s chi-square test).

**Figure 4.** Survival rates of patients with conventional renal cell carcinoma in relation to the level of CD44s expression: circle – grade 0; square – grade 1; triangle – grade 2; and asterisk – grade 3; p=0.003 (Kaplan-Meier).

Discussion

Our study showed the upregulation of CD44s molecule in 40.5% of conventional renal cell carcinoma specimens and the association of CD44s expression with adverse prognostic parameters, including poor cancer-specific survival. Normal renal parenchyma was negative for CD44s and all isoforms tested, except for some tubular epithelial cells in the close vicinity of the tumor tissue. This can be explained by the upregulation of CD44s, which is known to occur on the injured tubular cells (13,14). The expression of CD44s strongly correlated with nuclear grade and tumor stage, which are usually considered as the main prognostic factors in conventional renal cell carcinoma (3). Our finding that CD44s expression increased with the histological grade indicate a role of CD44 in tumor cell differentiation, as already postulated by others studies (15-18), although Gilcrease et al could not find this correlation (19). We, could not find the association between the expression of CD44 and tumor size, observed in some (17,18) but not in all studies (15). It is probably due to a generally smaller number of small tumors in our
study, compared to the tumor samples in other series. We have found reduced CD44s expression in tumors confined within the kidney, compared to pT3/4 tumors, as described by others (15,17-19). Quite contrary result regarding the relationship between CD44s expression and pathological tumor stage was reported by Hara et al (20) but using different methodology. They investigated the expression pattern of CD44 isofoms in 60 nonpapillary renal cell carcinoma samples and 15 normal kidneys by RT-PCR and found CD44s molecule expressed in normal kidneys and predominantly low-stage renal cell carcinoma. However, due to the fact that tissue samples consist of heterogeneous cell population, information about the mRNA for a specific protein molecule is less relevant than protein expression and its tissue localization. Therefore, it is difficult to draw conclusions from RT-PCR data, because false positive results may be generated by stromal lymphocytes of macrophages, which also express CD44 molecule.

In our study, CD44s strongly correlated with tumor cell proliferation expressed by Ki-67 labeling index, as described by Rioux-Leclercq et al (21). It is well known that the expression of Ki-67 is associated with a high rate of cell proliferation. Epithelia undergoing proliferation and cells under repair appear to upregulate both CD44 and hyaluronan production (22). One way by which CD44 molecule might influence cell proliferation is via interactions with regulatory factors. It has been shown that type I receptor tyrosine kinase signaling may be mediated by the CD44 family of transmembrane glycoproteins and that CD44 colocalizes with erbB-2 and epidermal growth factor receptor (EGFR) in erbB-2 positive metastatic mammary carcinoma cell lines (23). Moch et al (24) described the relationship between EGFR-receptor expression and Ki-67 index in renal cell carcinoma and their association with a poor prognosis. In the light of these findings, it would be interesting to analyze the relationship between Ki-67 index, EGFR, and CD44 molecule in renal cell carcinoma.

There are contradictory results regarding the expression of particular splice variants in renal cell carcinomas. Therpe et al (16) reported that no expression of CD44 variant exons was detected in normal kidneys, and there was a significant increase in the expression of CD44s and variant isoforms containing v6 and v9 exons in the course of tumor differentiation in conventional renal cell carcinoma. These findings are consistent with our data demonstrating strong correlation of v6 and v5, variant isoforms, with tumor differentiation. Heider et al (25) detected the expression of CD44 isoforms containing variant exon v5, v7, v8, and v10, but not v6, in most clear cell renal cell carcinomas. However, the number of cases in their study was rather small and included only 27 renal cell carcinomas. In the study of Paradis et al (17), only 2 out of 66 conventional renal cell carcinoma displayed v6 positivity, similar to the findings of Gilcrease et al (15), whereas Laurent et al (18) could not detect any v6 positive tumor in a series of 95 localized conventional renal cell carcinoma. Divergent conclusions regarding their pattern of expression and the role of CD44 isoforms in the process of tumor progression could be explained by a different methodological approach, different sensitivity and specificity of the antibodies used in the studies, and variations in antigen-retrieval methods. In addition, the assessment of positivity and the scoring systems are different between the studies. Regarding the need for antigen retrieval in paraffin sections, we found superior staining results by boiling the slides in a pressure cooker, as described previously (26), compared to other tested heat-induced antigen retrieval methods. Therefore, there is an obvious need for standardization in the experimental design to achieve comparable results.

Concerning the prognostic significance of CD44, our study corroborated the findings of Paradis et al (17), since we also found the overexpression of CD44s significantly associated with patients survival in univariate analysis. However, contrary to this study, no significant correlation was revealed by multivariate analysis. The strong CD44s expression as a predictor of tumor-related death was also documented by others (18,21).

The exact role of CD44 molecule in the progression of human tumors is still unknown, although several mechanisms are proposed. It has been shown that the binding of CD44 molecule to hyaluronan affects cell adhesion to extracellular components and is implicated in the stimulation of aggregation, proliferation, and migration. However, although it is clear that CD44 functions as a cell adhesion molecule, there is also substantial evidence that it is also a potent signaling receptor (27). The intracellular domain of CD44 selectively interacts with cytoskeletal proteins and regulates specific signaling pathways leading to the onset of multiple functions such as related cell adhesion, proliferation, migration, and invasion. Related invasion could be mediated by the upregulation of proteolytic enzymes (28). As a proteoglycan, CD44 can present and bind growth factors and chemokines leading to increased related growth (29). Also, the role of CD44 molecule in neoangiogenesis has been reported (30).

In conclusion, our findings support the hypothesis that the overexpression of CD44s is associated with the progression of conventional renal cell carcinoma. Although without an independent value in multivariate analysis, it may be used as an additional adverse prognostic factor in conventional renal cell carcinoma patients.

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References


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