**Immunohistochemical Markers for Metastasis in Clear Cell Renal Cell Carcinoma**

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**Background**: Renal cell carcinoma (RCC) is notorious for its high metastatic potential, and 30% of RCC patients present with metastatic disease at the initial presentation and 50% of them will develop metastasis or recurrence after radical surgery. **Methods**: In an attempt to identify the best predictive marker(s) for metastasis in patients with clear cell RCCs (CRCCs), we examined the expression patterns of 7 metastasis/prognosis-related markers by constructing a tissue microarray including primary CRCC specimens from 30 metastatic and 60 non-metastatic CRCCs. The markers we studied were Ki-67, MUC1, CD44s, PTEN, gelsolin, CA9 and p53. **Results**: The expressions of Ki-67, PTEN, CD44s, gelsolin and p53 were increased, whereas those of MUC1 and CA9 were decreased in the metastatic CRCCs compared with the non-metastatic CRCCs. The receiver operating characteristic curve-area under the curve (AUC) value of Ki-67 was 0.671, which was the highest among the 7 markers. The optimal cut-off value, sensitivity and specificity of the Ki-67 expression were 1.67%, 86.7% and 41.7%, respectively. **Conclusions**: These results demonstrate that the Ki-67 expression was increased in metastatic CRCCs, and it had the highest predictive value among the 7 markers. This suggests that Ki-67 could be an excellent predictive marker for metastasis in CRCC patients.

**Key Words**: Clear cell renal cell carcinoma; Metastasis; Ki-67

Renal cell carcinoma (RCC) is the most common malignant neoplasm of the kidney; it is the seventh leading cause of cancer death and it accounts for 4% of all the estimated malignancies in men in the USA. About one-third of RCC patients have metastatic disease at the time of diagnosis and 50% of the patients who undergo nephrectomy for a localized RCC are expected to relapse. Although patients with a localized RCC have a good prognosis, those with metastasis have an unfavorable outcome with a 5-year disease-free survival rate of less than 5% if they are not treated. Furthermore, metastatic RCC is known to be resistant to conventional chemo/radiotherapy. Although immunotherapy with high-dose interleukin-2 has been shown to have a 14% overall objective response rate, the response is usually restricted to patients with a good performance status. Prediction of metastasis preoperatively or even postoperatively for the patients with a good performance status may help them benefit from immunotherapy. Yet tumor metastasis from RCC cannot be accurately predicted according to the tumor stage and grade. Therefore, an adjunctive immunohistochemical marker(s) might allow prediction of metastasis in patients with RCC. Several properties are required for a tumor to establish metastasis: active proliferation, adhesion to and invasion of the basement membrane and vessels, passage through the extracellular matrix and escape from antitumor immune surveillance. Among the molecules involved in these processes, those related to the poor survival, recurrence or metastasis of RCC include high expressions of Ki-67, a member of the mucin family mucin-1 (MUC1), p53 and the adhesion molecule CD44s, and low expressions of carbonic anhydrase 9 (CA9), the actin-binding protein gelsolin and the tumor-suppressor gene product phosphatase and tensin homolog (PTEN).

To identify an immunohistochemical marker(s) for metastasis in clear cell RCCs (CRCCs), which is the most common subtype with a poor prognosis, we analyzed the immunohistochemical...
cal expression patterns of 7 metastasis/prognosis-related markers in primary CRCCs with and without metastasis by using a tissue microarray construct.

**MATERIALS AND METHODS**

**Patients**

This study was approved by the Asan Medical Center Institutional Review Board. This retrospective study included 90 CRCC patients who underwent nephrectomy from March 1997 to September 2003 at Asan Medical Center and they had at least 3 year follow-up. These 90 patients were composed of 30 metastatic CRCC patients and 60 randomly selected non-metastatic CRCC patients. The clinical information was obtained by reviewing the patients’ medical records and radiological findings. The tumors were staged according to the 2002 tumor node metastasis (TNM) staging system proposed by the American Joint Committee on Cancer. The histological slides of all cases were reviewed for diagnostic reassessment according to the 2004 World Health Organization Tumor Classification and the Fuhrman nuclear grading system. Immunohistochemistry

Tissue microarray constructs with 1-mm-diameter cores were created from the formalin-fixed paraffin-embedded tissues of primary CRCCs and the corresponding non-neoplastic normal kidneys. Each case included three cores from the highest grade area that was representative for the Fuhrman nuclear grade of the tumor. The construct also included one core from normal renal cortex and another one from normal medulla.

Immunohistochemical staining was performed with using an autostainer (Ventana Medical Systems, Inc., Tucson, AR, USA). The primary antibodies used in this investigation were Ki-67 (1:100 dilution, Invitrogen Co. Carlsbad, CA, USA), MUC1 (1:200 dilution, Novocastra, UK), PTEN (1:100 dilution, Invitrogen Co., Carlsbad, CA, USA), CD44s (1:50 dilution, Chemicon international Inc, Temecula, CA, USA), gelsolin (1:4,000 dilution, Sigma Chemical Company, UK), CA9 (1:4,000 dilution, Novus Biologicals, Littleton, CO, USA) and p53 (1:300 dilution, Dako, Glostrup, Denmark). An endogenous biont blocking kit was used to reduce the nonspecific immunopositivity (Ventana Medical Systems Inc., Tucson, AR, USA). Diaminobenzidine was used as a chromogen and the tissues were counterstained with hematoxylin.

All the tissue microarray slides were evaluated by two independent pathologists, and both of whom were kept “blind” to the clinical and pathological information. The expression patterns of the molecules were recorded as the average percentages of positive cells in all the cores, except for Ki-67 for which the level was evaluated in the most active area.

**Statistical analysis**

The data was analyzed by SPSS 12.0K software. Crosstabs, Pearson’s $\chi^2$ test, Fischer’s exact test, the Kruskal-Wallis test and binary logistic regression analysis were used when appropriate. The receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to analyze the variables validity for use as metastasis predictive markers. The ROC curve analysis was also used to calculate the sensitivity and specificity with 95% confidence intervals (CI). Metastasis-free survival was tested by the Kaplan-Meier method. Differences were regarded as statistically significant at p values <0.05.

**RESULTS**

**Clinicopathological features**

The clinicopathological features of the metastatic and non-metastatic CRCCs are summarized in Table 1. There were no differences in age, gender, operative methods, tumor size and stage, Fuhrman nuclear grade and the status of lymphovascular invasion between the two groups. The patients were aged from 28 to 86 years (median age: 57.5 years) with a male to female ratio of 2.9:1. Radical nephrectomy was performed in 88 cases (98%) and partial nephrectomy was done in 2 cases (2%), with clear resection margins in all the cases. The tumors measured from 1.6 to 19.0 cm at the greatest dimension (mean size: 6.2 ± 3.1 cm).

Among the 30 metastatic CRCCs, metastasis was detected at the initial presentation in 16 cases (53%) and at 4-54 months post-operation (median: 14 months) in the others. Twenty-six metastatic cases presented with solitary organ metastasis and the other 4 cases presented with multiple organ metastases. The most frequent single metastatic site was the lung (14 cases), followed by bone (11 cases) and brain (1 case). The cases of multiple organ metastases were 1 case each of lung and liver, lung and lymph node, bone and liver, and bone and lymph node.
Lymph-node dissection was performed in 33 cases (55%) of the non-metastatic CRCCs and in 16 cases (53%) of the metastatic CRCCs, and 2 of the latter cases had nodal metastasis. The cases of metastatic CRCC had a shorter follow-up period compared to that of the cases of non-metastatic CRCC (p<0.001).

Expression patterns of the markers in normal kidneys and CRCCs

Ki-67 was not expressed in most of the normal kidneys, but its expression was noted in the nuclei of the tubular epithelial cells where inflammatory cell infiltration was present. The Ki-67 expression was mildly increased in the CRCCs (mean: 4.2 ± 4.5%) (Fig. 1D-F). MUC1 was expressed in the apical cytoplasmic membrane of the collecting ducts and distal tubules in the normal kidneys. Some collecting ductal cells revealed strong cytoplasmic immunopositivity (Fig. 1G). The CRCCs also showed a MUC1 expression in the apical membrane of the tumor cells (Fig. 1H, I). PTEN was strongly expressed in all the tubular segments and it was weakly expressed in the glomeruli of the normal kidneys (Fig. 1J). The PTEN expression was markedly decreased in the CRCCs, although the non-neoplastic stromal cells were strongly positive for PTEN (Fig. 1K, L). The CD44s expression was similar to the Ki-67 expression in the normal kidneys: there was no expression in most cases, but there was a membranous expression in the tubules adjacent to the inflammatory cell infiltration (Fig. 1M). CD44s was not expressed in most of the CRCC cases (74 cases, 82%), but 16 cases (18%) showed membranous immunopositivity (Fig. 1N, O). Gelsolin was strongly expressed in the cytoplasm or cytoplasmic membrane of the distal tubules and collecting ducts of the normal kidneys, and it was weakly expressed in the CRCCs as well (Fig. 1P-R). No CA9 expression was noted in the non-neoplastic kidneys (Fig. 1S). In contrast, CA9 was diffusely expressed in most of the CRCC cases (82 cases, 91%) as a membranous pattern (Fig. 1T, U). There were a few scattered p53 positive tubular cells in the non-neoplastic kidneys and its expression was mildly increased in the CRCCs (5.6 ± 16.3%) (Fig. 1V-X).

Expression patterns of the markers between the non-metastatic CRCCs and the metastatic CRCCs

The expressions of Ki-67, PTEN, CD44s, gelsolin and p53 were increased, whereas those of MUC1 and CA9 were decreased in the metastatic CRCCs compared with the non-metastatic CRCCs. According to the ROC curves analysis, among the 7 proteins, Ki-67 was the only protein that had an AUC value greater than 0.6 (Table 2). The optimal cut-off value for the Ki-67 expression to discriminate metastatic CRCC from non-metastatic CRCC was 1.67%, which revealed a sensitivity of 86.7% (95% CI: 69.3-96.2%) and a specificity of 41.7% (95% CI: 29.1-55.1%). The estimated crude odds ratio (OR) of the association between the Ki-67 expression with the cut-off value 1.67% and metastasis was 4.643 (95% CI, 1.439-14.976, p=0.01). When adjusted for the tumor size, stage and Fuhrman nuclear grade, the adjusted OR was 4.389 (95% CI: 1.314-14.659, p=0.016). Those CRCCs that were expressing Ki-67 at a higher level than the cut-off value (1.67%) revealed a reduced rate of metastasis-free survival (p=0.01) (Fig. 2).

DISCUSSION

The single most important factor affecting survival in CRCC is the presence of metastatic disease. It has been suggested that the expressions of Ki-67, MUC1, PTEN, CD44s, gelsolin, CA9 and p53 in CRCCs were related to the survival of CRCC patients, but their value as predictive markers of metastasis has been a matter of debate. Here, we compared the expressions of the markers in primary tumors of metastatic and non-metastatic CRCCs.
Fig. 1. Expression patterns of the 7 proteins in non-neoplastic normal kidneys and clear cell RCCs. The first column shows representative images of non-neoplastic kidneys (A, D, G, J, M, P, S, and V), the second column non-metastatic clear cell RCCs (B, E, H, K, N, Q, T, and W) and the third column metastatic clear cell RCCs (C, F, I, L, O, R, U, and X). The first row (A, B, and C) is stained by hematoxylin-eosin staining (H&E) and the others (D-X) by immunohistochemical staining.
and we demonstrated that Ki-67 was the best immunohistochemical marker, among the 7 markers that were analyzed in this study, to predict metastasis in CRCC patients.

There have been many attempts to predict the survival of RCC patients, and this has resulted in developing multivariate prognostic systems.\(^{10-12}\) They combine the important clinical and pathological parameters, including the T stage, metastasis, performance status, nuclear grade and coagulative necrosis of the tumor. It has been noted that the addition of immunohistochemical markers yields a better prediction of disease-specific survival, which proves the value of immunohistochemical markers as prognostic factors.\(^{13}\) For example, the University of California Los Angeles integrated staging system shows that the poor disease-specific survival of RCC patients is associated with the high expression levels of vimentin, gelsolin and p53, and the low expression levels of CA9 and PTEN, in addition to the presence of metastasis, a high T stage and a high Eastern Cooperative Oncology Group performance status.\(^{10}\) High incidence rates of metastasis have also been reported in patients with high expression levels of Ki-67, MUC1 and CD44s.\(^{14,15}\)

The high expression of Ki-67 was well known as a poor prognostic marker in terms of the survival, recurrence and metastasis of RCC, and even when the analysis was restricted to CRCC.\(^{11,14,16,17}\) In agreement with the previous reports, our study also showed a higher Ki-67 expression in metastatic CRCCs compared with that in the non-metastatic CRCCs. To the best of knowledge, the present study is the first to systematically study the 7 metastasis/prognosis-related markers in metastatic and non-metastatic CRCCs, and to demonstrate that among them, Ki-67 is the best immunohistochemical marker to predict metastasis in CRCCs.

Although previous reports have shown that a higher MUC1 expression was related to a high incidence of metastasis, the MUC1 expression was decreased in the metastatic CRCCs in our study.\(^{15}\) MUC1 is a heavily glycosylated membrane protein that is normally expressed at the luminal surface of many glandular and ductal epithelia, including the collecting ducts and distal tubules in the normal kidney.\(^{18}\) It is an anti-adhesion molecule and its generally known to be an indicative of a poor prognosis.\(^{15}\) However, a study using an RCC cell line that was stably transfected with MUC1 cDNA revealed a slower growth rate in vitro, and lower tumorigenicity and a lower metastatic potential in vivo than the mock-transfected cells, which suggests an inhibitory role for MUC1 on the tumorigenesis and metastasis of RCC.\(^{19}\) Our results showing a reduced MUC1 expression in metastatic CRCCs support the inhibitory role of MUC1 on metastasis.

The search for factors that are predictive of metastasis in RCC is in progress and new markers continue to be identified. They include L1 cell adhesion molecule, one of the major structural components of caveolae caveolin-1, an oncofetal RNA-binding protein IMP3, an apoptosis inhibitor survivin, a polyamine Spm8-2 and a T-cell co-stimulatory molecule B7-H1.\(^{20-25}\)

### Table 2. Expression of the 7 proteins in non-metastatic CRCCs and metastatic CRCCs

<table>
<thead>
<tr>
<th></th>
<th>Non-metastatic CRCCs*</th>
<th>Metastatic CRCCs*</th>
<th>Total*</th>
<th>AUC' (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>3.6 ± 4.7</td>
<td>5.4 ± 4.2</td>
<td>4.2 ± 4.6</td>
<td>0.671 (0.56-0.766)</td>
</tr>
<tr>
<td>MUC1</td>
<td>20.4 ± 27.2</td>
<td>13.0 ± 20.4</td>
<td>17.9 ± 25.3</td>
<td>0.566 (0.457-0.670)</td>
</tr>
<tr>
<td>PTEN</td>
<td>2.8 ± 13.9</td>
<td>6.6 ± 18.3</td>
<td>4.1 ± 15.5</td>
<td>0.559 (0.450-0.663)</td>
</tr>
<tr>
<td>CD44s</td>
<td>2.7 ± 10.4</td>
<td>10.1 ± 21.5</td>
<td>5.1 ± 15.3</td>
<td>0.597 (0.489-0.699)</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>6.1 ± 18.9</td>
<td>8.9 ± 20.9</td>
<td>7.0 ± 19.5</td>
<td>0.544 (0.436-0.650)</td>
</tr>
<tr>
<td>CA9</td>
<td>78.5 ± 32.9</td>
<td>69.4 ± 35.8</td>
<td>75.5 ± 33.9</td>
<td>0.588 (0.479-0.691)</td>
</tr>
<tr>
<td>p53</td>
<td>4.2 ± 12.4</td>
<td>8.5 ± 22.2</td>
<td>5.6 ± 16.3</td>
<td>0.519 (0.411-0.626)</td>
</tr>
</tbody>
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* mean percentage ± standard deviation; ′, value of area under an ROC curve (AUC) with the range of 95% confidence interval.

CRCCs, clear cell renal cell carcinoma.

![Fig. 2. Metastasis-free survival of clear cell RCC patients according to Ki-67 expression. The metastasis-free survival was reduced when Ki-67 expression was high with a cut off value of 1.67%](image)

The table above summarizes the expression levels of the 7 proteins in non-metastatic and metastatic CRCCs. The table includes the mean percentage ± standard deviation, along with the area under the ROC curve (AUC) with the range of 95% confidence interval.
Since most of the previous reports have studied only one or two molecules, we intended to systematically evaluate metastasis/prognosis-related proteins to select the best predictor of metastasis. Our results indicate that Ki-67 is the best marker among the 7 markers we tested. However, the AUC value of the Ki-67 expression was only 0.671, which shows that this marker has poor discriminative value to predict metastasis in CRCC patients. Therefore, further studies that will include newly discovered markers might help identify better markers for predicting metastasis in CRCC patients.

REFERENCES