Prognostic Value of CD44 and Ki-67 in Renal cell carcinoma

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Received 9 March 2014  Accepted 30 September 2014

Abstract
Renal cell carcinoma accounts for approximately 3% of adult malignancies and 80 - 90% of all primary malignant renal tumors. The origin of renal cell carcinoma is from the proximal renal tubular epithelium. Tumor stage and nuclear grade were considered as the most important prognostic variables for patients with renal cell carcinoma but they are insufficient to predict the clinical behavior of this tumor. Ki-67 is a nuclear protein that is widely accepted as a reliable indicator of cell proliferation and has a demonstrable utility as a prognostic marker for several malignancies. The cluster of differentiation 44 (CD44) is a transmembrane glycoprotein that involved in cell-cell and cell-matrix interactions; and its expression has been linked to tumor cell invasion and tumor metastasis in several cancers.

The objective of this study is to provide a better understanding of the biology of renal cell carcinoma by: Evaluation of the expression of CD44 and Ki-67 in renal cell carcinoma. Correlate their expression with the usual prognostic variables (tumor size and stage and nuclear grade). Thirty cases of nephrectomy for renal cell carcinomawere included in this retrospective study. All formalin fixed and paraffin embedded tissues were retrieved from the pathology department of Al- Harreri surgical specialties teaching hospital and teaching Laboratories in the Medical City during the period from January 2010 to March 2013. Hematoxylin and eosin sections were prepared by routine technique and also 3 µm thickness sections were stained immunohistochemically for CD44 and Ki-67.

The clear cell renal cell carcinoma was the most common histological subtype (86.7% of the cases). The expression of ki-67 was positive in twenty five of cases (83.3%) while CD44 was expressed in twenty seven of cases (90%) out of thirty. The expression of CD44 and Ki-67 was increase progressively with increase nuclear grade and tumor stage. The expression of CD44 and Ki-67 showed weak correlation with tumor size and there was no significant correlation with age, gender and histological type of tumor. The expression of CD44 and Ki-67 were significantly associated with tumor stage and nuclear grade of renal cell carcinoma. CD44 and Ki-67 can be used as an additional adverse prognostic factor in patients with renal cell carcinoma.

Key words: Renal tumor, CD 44, KI67, Renal cell carcinoma, Immunohistochemistry.

دراسة مرضية نسيجية لسرطان الخلايا الكلوية بواسطة العوامل الكيميائية المناعية CD44 و Ki-67

الخلاصة

ان سرطان الخلايا الكلوية يشكل تقريبا 3% من سرطان البالغين و 80 - 90% من الأورام الأولية الخبيثة للكلية. ان منشأ سرطان الخلايا الكلوية هو من ظاهرة الإندوب الكلوي الأقرب. ان مرحلة الورم ودرجة النواة يعتبران اهم المتغيرات التكنئيية لمرضى سرطان الخلايا الكلوية، لكنهما غير كافيين لتوقع السلوكي السريري لهذا الورم. 67-Ki هو بروتين نووي مقبول بشكل واسع كمؤشر موثوق به للكئات الخلايا. له ساحة واضحة كمجم كيميائي لعده اورام خبيثة. CD44 هو كلاكيوبروتين عبر.
Renal cancer is a heterogeneous disease consisting of various subtypes with diverse genetic, biochemical, and morphologic features. Renal cell carcinoma accounts for the vast majority of renal malignancies in adults [1]. It originates from the lining of the proximal convoluted tubule [2]. This disease is characterized by a lack of early warning signs, diverse clinical manifestations, and resistance to radiation and chemotherapy [3]. The classic clinical presentation of flank pain, hematuria, and palpable flank mass is comparatively uncommon (5-10% of cases) [4]. Cigarette smoking and obesity are considered as the leading cause and strongest known risk factors of RCC. Hypertension and family history of the disease are also risk factors [5]. Patients with certain inherited disorders such as von Hippel-Lindau disease show an enhanced risk of renal cell carcinoma [6, 7, 8, 9]. It has been suggested that Ki-67 having prognostic value in renal cell carcinoma CD44 is a trans membrane glycoprotein involved in cell–cell and cell-matrix interactions; it represents a large family of cell-adhesion molecules, which differ mainly in primary structure, with a predominant form (CD44H, standard or hematopoietic form) and several variant isoforms [10,11,12,13]. CD44 has been reported to play an important role in cancer cell invasion and metastasis as well as in fundamental biological processes, including lymphocyte homing, inflammation, hematopoiesis, wound healing, and apoptosis [14]. As a molecule involved in mechanism of cell motility, CD44 has been used as a marker for tumor aggressiveness in a number of malignancies such as stomach, liver and breast. However, in renal cell carcinoma there are contradictory results, with some series supporting the role of CD44 as a prognostic marker while in other studies its importance as a predictor of survival was not confirmed [15].

The objective of this study is to provide a better understanding of the biology of renal cell carcinoma by evaluation of the expressions of CD44 and Ki-67 in renal cell carcinoma and to correlate their expression with the usual prognostic variables (tumor size, stage and nuclear grade).

Materials and Methods
Patients
Between January 2010 and March 2013, thirty patients (9 women and 21 men whom their age ranging between 25 and 78 years), who underwent radical nephrectomy for a primary RCC; were included in this retrospective study. Clinical data were
obtained from patient medical records in the archives of the pathology departments of Al-Harreri Surgical Specialties teaching hospital and the teaching laboratories of medical city. Histopathological reports and slides were available for all these cases. The macroscopic features, including tumor size and histological features including nuclear grade as defined by Fuhrman et al [86] were assessed. The histological subtype was assessed according to the consensus classification of RCC. Tumor stage was defined according to the International Union against Cancer and the American Joint Committee on Cancer TNM classification. All paraffin embedded sections were retrieved and re-stained with the routine H and E stain for verifying the morphologic diagnosis. All specimens were reevaluated for pathological stage, grade and histological subtypes and compared with previous pathological reports.

Materials:
Markers: The primary antibodies used in this study are listed in table (3.3).

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
<th>Type</th>
<th>Code number</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>Dako</td>
<td>Monoclonal Mouse Anti-Human CD44 protein</td>
<td>M7082</td>
<td>T&amp;B lymphocyte in the tonsil</td>
</tr>
<tr>
<td>Ki 67</td>
<td>Dako</td>
<td>Monoclonal Mouse Anti-Human Ki 67 protein</td>
<td>M7240</td>
<td>T&amp;B lymphocyte in the tonsil</td>
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Staining Kit:
Immunophosphatase secondary detection system (DakoCytomation LSAB+ System-HRP, Code K0673), sufficient for 150 tissue sections ,based on 100 microliter per section, which include:
1- Biotinylated secondaryantibody (BSA)
2- Streptavidin –peroxidase antibody.
3- Hydrogen peroxide.
4- chromogene working solution (AEC).

Methods
1. Deparaffinized sections were rehydrated and washed in buffer, phosphate buffer saline (PBS) for 5 minutes.
2- Protein blocking was done by removal of slides from buffer, wiped gently around each section, and covered each with protein blocking agent (PBA), we allowed to incubate for 5 minutes at room temperature.
3- Primary antibody: PBA were poured off, wiped gently around each section and covered the section with primary antibody. All sections Incubated at the humidity chamber at room temperature for 30-60 minutes, depending on the antibody.
4- All sections then were washed with three changes of the buffer for 2 minutes each.
5- Application of secondary antibody were done by wiping gently around each section and covering each section with biotinylated secondary antibody. Followed by incubation in a humidity chamber for 10 minutes at room temperature.
6- wiped around section and covered each section wit streptavidin-peroxidase reagent this followed by incubation in humidity chamber for 10 minutes
7- wiped around each section and covering each section with a freshly prepared Chromogene solution followed by incubation for 10 minutes at room temperature.
8- Slides were washed with water for 2 minutes.
9- covered each section with hematoxylin incubate for 1 to 2 minutes then washed in running water for 2 minutes.
Covering AEC stained slides with an aqueous mounting medium was done. Interpretation of the staining results:

A positive stain is indicated by the brown colored precipitate at site of specific cellular antigen localization. Positive controls (tonsil) were included in each run. The evaluation of the immunohistochemical preparation of CD44 was performed semiquantitatively and expressed as percentages of positive cells by counting at least 1000 tumor cells at medium magnification (X100). Positive staining for CD44 was defined as a membranous and/or cytoplasmic staining pattern of epithelial tumor cells, even if the staining was focal in tumor cells. The immunohistochemical results for CD44 were scored according to four grades as follows: - No positive cells (score 0)

- Fewer than 25% reactive cells (score 1)
- Between 25% and 75% positive cells (score 2)
- More than 75% positive cells (score 3) [15].

Ki-67 antigen labeling was localized to the nucleus with a fine, strong and homogenous brown granularity. Staining was considered positive if any nuclear staining was seen. Evaluation was performed by counting approximately 1000 nuclei of tumor cells per slide. Ki-67 labeling index was derived by dividing the number of positively stained nuclei by the total number of nuclei counted in areas of maximal proliferation that were identified at medium magnification (X100), both stained and non stained nuclei multiplied by 100. Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. Statistical analyses were done using SPSS version 20 computer software (Statistical Package for Social Sciences) and Microsoft Office excel 2010. Associations between 2 categorical variables was explored by cross-tabulation. The Chi-square test and Fisher exact test were used to verify the association between CD44 and Ki-67 expressions and clinicopathological variables. The statistical significance, direction and strength of linear correlation between 2 quantitative variables, was measured by Spearman’s linear correlation coefficient. P value less than the 0.05 level of significance was considered statistically significant.

Results

Thirty cases of RCC were included in this retrospective study. The patients' ages ranged from 25 to 78 years, with a mean of age was 53.5 years and the peak age of incidence was at 50-69 years (40.0% of cases). Twenty one cases were men and nine cases were women with male-to-female ratio was 2.33:1 (70% male; 30% female). The mean of tumor size was 8 cm (ranging from 2 to 17 cm in its largest diameter. Twenty six of the RCC cases were of the conventional clear cell type (86.7%). The remaining cases included two papillary (6.7%), one chromophobe RCC (3.3%) and one case of carcinoma of collecting duct (3.3%).

The grade distribution of RCC was as follows: four cases of Grade 1 (13.3%), fifteen cases of Grade 2 (50.0%), eight cases of Grade 3 (26.7%), and three cases of Grade 4 tumors (10.0%). The expression of proliferative marker Ki-67 was identified by nuclear reactivity of <10% of cells (which is considered as negative) in five cases (16.7%) and >10% of cells in twenty five of cases (83.3%) which are regarded as positive. (Table-4.2). Comparisons of clinicopathological parameters by the mean of Ki-67 percent of positive tumor cells. There was obvious progressive increase in mean of Ki-67 percent of positive tumor cells with increase tumor grade and stage which was statistically significant (P value <0.001) and
there was very strong positive linear (direct) correlation between the mean of Ki-67 and the tumor grade and stage \((r=0.907 \text{ and } 0.722\) respectively). There was weak but not significant correlation between Ki-67 labeling index and tumor size \([r=0.378, \text{P}=0.04]\). There was no statistically significant association between mean of Ki-67 percent of positive tumor cells and age, gender and histological subtype of RCC \((\text{P value } >0.001)\). Regarding the expression of CD44, normal renal parenchyma was negative for CD44. RCC showed heterogeneous staining pattern of CD44. Most clear cell RCC with CD44 positivity showed a membranous staining pattern whereas papillary RCC generally showed combinations of membranous and cytoplasmic dot-like positivity in the paranuclear region and some tumors showed only focal positive staining of tumor cells. CD44 was expressed in twenty seven out of thirty cases (90%): thirteen cases (43.3%) of score I, eight cases (26.7%) of score II and six cases (20%) of score III. The expression of CD44 was compared with the usual clinicopathological parameters, including age, gender, tumor size, histological subtype, Fuhrman’s nuclear grade, pathological stage, and Ki-67 proliferation index. The median score of CD44 expression was the lowest in cases with stage I and grade I tumors (median score I) and highest among cases with high tumor stage (III and IV) and grade IV (median score II and III respectively). So there was a statistically significant association \((\text{P value } <0.001)\) and strong positive linear correlation of the CD44 median score with TNM staging and grade of tumor \((r=0.645\) and 0.755 respectively). The score of CD44 expression is progressively increased with the increase of the stage and grade of RCC. There was weak but not significant correlation between CD44 expression and tumor size \([r=0.223, \text{P}=0.24]\). Age, gender and histological subtype showed no statistically significant association with median score of CD44 \((\text{P value } >0.001)\). We also found very strong statistical correlation between CD44 expression and tumor growth fraction expressed as Ki-67 proliferation index \((\text{P}<0.001 \text{ and } r=0.811)\). The mean value of Ki-67 index in CD44 negative tumors was 9.0% whereas it was significantly higher in the group of score 3 CD44 positive tumors and reached 22%. Namely, the CD44 expression is significantly increase with increasing proliferative activity of tumor cells. Ki-67 had slightly higher validity \((\text{ROC}=0.921)\) than CD44 \((\text{ROC}=0.891)\) \((\text{P value } 0.001)\) in predicting advanced tumor stage, and both of them had a higher validity than tumor size \((\text{ROC}=0.755)\). Ki-67 was slightly more valid \((\text{ROC}=0.998)\) than CD44 \((\text{ROC}=0.931)\) \((\text{P value } 0.001)\) in predicting tumor of high grade and both of them showing higher validity than tumor size \((\text{ROC}=0.622)\) \((\text{P value } 0.027)\) demonstrate that the most sensitive \((100\%)\) cut off value for ki-67 percent of positive tumor cells to predict cases with advanced tumor stage (III-IV) was \(\geq 12.5\%\). Testing negative \(<12.5\%) at this highly sensitive cut-off value would exclude a high stage tumor with 100% confidence. The cut-off value of Ki-67 percent of positive tumor cells associated with a highest specificity \((100\%)\) was 21.5%. Testing positive at this highly specific cut-off value would establish the diagnosis of a high stage tumor with 100% confidence. The most sensitive \((100\%)\) cut off value for Ki-67 to predict cases with high tumor grade (III-IV), was \(\geq 17.5\%). Testing negative \(<17.5\%) at this highly sensitive cut off value would exclude a high grade tumor with 100% confidence. The cut off value of Ki-67 to predict cases with high tumor grade (III-IV) associated with highest specificity \((100\%)\) was 18.5%. Testing positive at this highly specific cut-off value would established the diagnosis of a high grade tumor with 100% confidence the most sensitive \((100\%)\) cut off value for CD44 score to predict cases with
high tumor stage (III-IV), was score I. Testing negative (<score I) at this highly sensitive cut-off value would exclude a high stage tumor with 100% confidence. The cut-off value of CD44 to predict cases with high tumor stage (III-IV) associated with highest specificity (94.4%) was scoring III. Testing positive at this highly specific cut-off value would establish the diagnosis of a high stage tumor with 98.5% confidence., the most sensitive (100%) cut-off value of CD44 score to predict cases with high tumor grade (III-IV), was score II. Testing negative (<score II) at this highly sensitive cut-off value would exclude a high grade tumor with 100% confidence. The cut-off value of CD44 to predict cases with high tumor grade (III-IV) associated with highest specificity (94.7%) was scoring III. Testing positive at this highly specific cut-off value would establish the diagnosis of a high grade tumor with 98.7% confidence.

**Discussion**

Tumor stage and nuclear grade are usually considered as the main pathological prognostic factors, but improved prediction is needed and attempts to find better prognostic criteria remain under investigation. Immunohistochemical markers will eventually enhance our ability to predict the behavior of an individual tumor and to stratify patients into more sophisticated risk categories, ultimately permitting the goal of moving from nonspecific treatments to designing and targeting therapies for targeted patient populations [15, 16].

In the present study the prognostic value of classical clinical, pathological and some immunohistochemical variables in tissue specimens of RCC was evaluated retrospectively in thirty patients. In our study, the mean age of patients was 53.5 years which was parallel to that in the study of Mahmood et al [17] in which the mean age was 52.64 years, and Yildiz et al [13] where the mean age was 54 years. The age of the patients in our study was significantly lower in compare to the Japanese study done by Li N. et al. (the mean age 62 years) [18]. Also in western countries RCC is a disease of elderly patients, as in the study of Gilcrease M Z et al. where the mean age is 65 years [19].

Our study revealed that the male-to-female ratio was 2.33 (70% male; 30% female) which is significantly higher in compare to that of Iraqi cancer registry 2005 in which the ratio was 1.60 [19] and also higher than that in the study of Mahmood et al [17] and in the study of Li. et al [18] where the male-to-female ratio was 1.48 and 1.78 respectively. The most common type of RCC in the present study was conventional type (86.7%) that was higher than that in study of Mahmood et al [20-21], the Japanese study done by Li N. et al [108] and in the study of Gilcrease M Z et al [10] where the conventional clear RCC form 76.08%, 81.25% and 72.09% of cases respectively. This difference in age and sex distribution and in the percentage of most common type of RCC may be due to small sample size in the present study (thirty cases) and bias in selection of the cases. Cell proliferation is the simplest and most commonly used variable in evaluating tumor progression and prognosis, and the expression of Ki-67 has been accepted as an excellent indicator of tumor proliferation. In our study, 83.3% of cases show positivity for ki-67. The positivity rate of ki-67 in the present study is parallel to that noticed in the study of Wafaa Helmi et al. (80%) [23-25].

In the present study, the reactivity for Ki-67 was heterogeneous in the same tumor, which is similar to that noticed in the study of Wafaa Helmi et al. and study the proliferation index expressed by ki-67 was significantly correlate with advanced clinical stage, high nuclear grade of RCC, this was parallel to that found by Wafaa Helmi et al [109], Matthew K. et al, and Rioux-Leclercq N et al [24-26]. Weak correlation was found in the present study between Ki-67 expression and tumor size, on the contrary,
there was significant correlation between larger tumor size and Ki-67 expression by Matthew K et al [27], and Rioux-Leclercq N et al although this association was not observed by Wafaa Helmi et al. There was no statistically significant association between mean of Ki-67 percent of positive tumor cells and age, gender and histological subtype (P value >0.001). The CD44 is an adhesion molecule involved in cell-matrix interaction and its expression has been linked to tumor metastasis in several cancers. There are limited data regarding the expression of CD44 in RCC and many of the results are contradictory. Our study showed expression of CD44 molecule in 90% of RCC specimens and there was strong correlation of CD44 expression with adverse prognostic parameters, including tumor stage, grade and proliferative activity. We have found reduced CD44 expression in tumors confined within the kidney, compared to advanced stage tumors, these results were similar to that described by others (Paradis V et al [28], Gilcrease MZ et al [29]). Our findings were also include that the CD44 expression increased with the nuclear grade of RCC, as already postulated by others studies (Paradis et al; and Daniel L et al [30]). Our study showed weak correlation but not statistically significant association of CD44 expression in relation to the tumor size, this result was agree with that of Ksenija Luèin et al [31]. This discrepancy in correlation of tumor size with expression of both CD44 and Ki-67 was probably due to a generally small number of small size tumors in our study, compared to that in other series. In our study, there was also a strong statistical correlation between CD44 expression and tumor cell proliferation expressed by Ki-67 labeling index .The CD44 expression significantly increased with the increasing proliferative activity of tumor cells. Similar results were found by Ksenija Luèin et al [32-33]. Age, gender and histological subtype showed no statistically significant association with median score of CD44 (P value > 0.001).

**Conclusion**

The age incidence of RCC in general showing a peak through the period of 50-69 years and RCC was more common in male with male: female ratio equal to 2.33:1.Conventional clear cell RCC was the most common histological subtype (86.7%). The expression of CD44 and Ki-67 were significantly associated with advance tumor stage and high nuclear grade of RCC but show weak correlation to tumor size and no significant correlation with age, gender and histological type of RCC. In addition to standard clinicopathological parameters (tumor stage, tumor size, nuclear grade), Ki-67 is an easy and reliable marker that could be applied on formalin fixed tissue for better assessment of biological behavior of RCC and predicting patient outcome. The overexpression of CD44 is associated with the progression of RCC and it may be used as an additional adverse prognostic factor in RCC patients.

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