The Association of Immunoreactive p53 and Ki-67 with T-stage, Grade, Occurrence of Metastases and Survival in Renal Cell Carcinoma

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Abstract. Background: The aim of this study was to clarify the association of p53 and Ki-67 protein expressions with tumor characteristics and survival in renal cell carcinoma (RCC). Materials and Methods: One hundred and seventeen patients were included in the study, 101 (86%) with conventional RCC according to the Heidelberg classification. Patients were divided into three groups with either primary metastases (pm), later metastases (lm), or no metastases (nm) during 7.5 years follow-up. Paraffin-embedded tissues were examined by immunohistochemistry utilizing anti-p53 and anti-Ki-67 antibodies, with a positive reaction cut-off of 10%.

Results: In conventional RCC, there was more Ki-67 positivity in high T(tumor)-stage compared to low T-stage (p=0.036) and in pm patients compared to nm patients (p=0.007); p53 was not associated with T-stage or metastatic category. Co-expression of p53/Ki-67 was more common in pm patients than in lm patients, but was not observed in nm patients (p=0.001). In the pm/lm group, p53 and Ki-67 expressions were associated with decreased survival (log-rank, p=0.030 and p=0.031, respectively). In lm patients, high T-stage (T3, T4) was associated with metastases-free survival (p=0.034) and overall survival (p=0.006). Conclusion: p53 and Ki-67 expressions are associated with aggressive tumor phenotype and decreased survival in metastatic RCC. Ki-67 alone was a stronger prognostic marker than p53 for development of metastases. Double positivity for p53 and Ki-67 expression in RCC patients seems to indicate a high metastatic probability.

Since the 1970s, the annual increase in the incidence of renal cell carcinoma (RCC) has been between 2 and 4%, which has been explained by the use of imaging procedures and the increasing prevalence of etiological risk factors, such as tobacco smoking and obesity (1, 2). Half of the patients without metastases at diagnosis will develop recurrence of their cancer; two-thirds within the first year (3) and the majority within 5 years (4). The 5-year survival rate for patients with metastatic RCC increased from less than 10% to 15-30% during the last decade due to the advances in RCC therapy (5-9). In addition to nephrectomy, metastatic RCC is currently treated with biological response modifiers, such as interferon-α and interleukin-2 (5-8). One of the main areas of cancer research is to define the characteristics of patients at high risk of metastases, who could be targeted for therapeutic intervention (10, 11).

Performance status, time to metastasis, site of metastases, prior nephrectomy and weight loss have prognostic value in RCC (6, 12). Currently, tumor size and grade are the most important prognostic factors known for the survival of patients with locally confined RCC (9, 13, 14). Even though many tumor grading systems have been developed, none has so far gained universal acceptance (15). The UICC pTNM classification system for renal carcinomas was updated in 1997 and revised in 2002 (16, 17). The Heidelberg classification was conceived in a workshop in 1996, when new genetic findings were linked to the knowledge of morphology (18). More specific prognostic markers, related to the molecular mechanisms of RCC, are needed to specify the diagnosis, staging and prognosis, and to guide targeted cancer therapies (19). Additionally, prognostic markers may identify patients needing more frequent follow-up. Therefore, in research on tumor biology, it is important to study the relationships of biomarkers to prognosis.

p53 is a tumor suppressor gene and p53 protein expression has been shown to be expressed in RCC (20). Mutant p53 has an extended half-life; it accumulates in cell nuclei and can be
immunostained, whereas wild-type p53 is usually undetectable by routine immunohistochemistry, because of its short half-life (21). In normal cells, p53 is usually undetectable (22). Activated p53 elicits several cellular responses, including apoptosis and cell cycle arrest (23). It responds to DNA damage at the restriction checkpoint of the G1-phase of the cell cycle (24). Ki-67 protein is expressed throughout the active phases of the cell cycle, and its expression is related to the proliferative activity in the cell nuclei (25); Ki-67 accumulates during the cell cycle from G1 to mitosis, at its lowest level after mitosis (26), and has been shown to be expressed in RCC (20).

The aim of the present study was to examine the associations between p53 and Ki-67 protein expressions with histopathological and clinicopathological parameters, as well as their prognostic value for survival in RCC. The study targeted three different groups of RCC patients: i) patients with primary metastases (pm); ii) patients who later developed metastatic disease (lm); and iii) patients with no occurrence of metastases (nm).

Materials and Methods

Patients, staging and histology. The study included samples from 117 patients with local or metastatic RCC treated in the Turku University Hospital, Finland. Consecutive samples were collected from patients with local RCC treated during 1986-1996 and with metastatic RCC during 1995-2001. As the patients with local disease had been free of metastases for at least 7.5 years, their samples were from an earlier period than the samples from patients with metastatic disease. All the 117 patients had undergone nephrectomy. Patients with metastatic disease were treated with interferon-α according to the schedule described in detail earlier (6). Radiological evaluations during follow-up were performed by chest X-ray and abdominal ultrasound at regular 6-month intervals after nephrectomy. Radiological evaluations of the tumor spread in interferon-α treated patients were performed by chest and abdominal CT scan or chest X-ray and abdominal ultrasound at regular 3-4 month intervals and the evaluation of the response followed the criteria of the World Health Organization (WHO) (27).

The 1997 updated UICC pTNM classification system of renal carcinomas for T-staging was used (16). Histopathological samples were re-evaluated by an experienced pathologist (KOS) who regraded the tumors according to the WHO classification (28), and categorized them according to the Heidelberg classification (18); 101 (86%) patients had conventional RCC (Table I).

The patients were divided into three groups according to the metastatic category: the first group had distant metastases at presentation (pm), the second group developed metastases (lm) during the follow-up of 76 (median) months (range 15-177), and the third group had no evidence of metastases during at least 7.5 years follow-up (nm). The total number of patients was 117, of whom 29 (25%) were in the pm group, 37 (32%) in the lm group, and 51 (44%) in the nm group.

Immunohistochemical staining and scoring of p53 and Ki-67. From paraffin-embedded blocks, 5-μm-thick sections were cut, deparaffinized with xylene and rehydrated through a graded series of alcohol. For antigen retrieval, the samples were boiled for 10 min in a microwave oven in 10 mM sodium citrate buffer (pH 6.0). The samples were then incubated with commercial monoclonal p53 antibody clone DO-7 (DAKO, Denmark) diluted to 1:300 and commercial monoclonal Ki-67 antibody (MB-1 antibody, DAKO) diluted to 1:100 for 27 min and were visualized by avidin-biotin-peroxidase staining. An automated processor (TechMate 500, DAKO) was used. The percentage of nuclei with immunoreactive p53 and Ki-67 was counted for each tumor slide by consensus of two investigators (MK, KOS). Immunoreactivity was classified as continuous data from undetectable levels (0%) to homogeneous (100%) for both markers. To avoid false positivity, the reaction was considered positive when 10% or more of the cancer cells showed staining. The 10% cut-off value was selected to achieve statistically reliable results, as well as according to a previous study on the subject (29). Staining without the primary antibody served as negative control. No significant background staining was detectable. The reliability of staining was measured by positive controls used as weekly standard controls in the routine pathology laboratory. Two positive and two negative stainings of p53 and Ki-67 protein expressions are shown in Figure 1.

Statistical analysis. Univariate associations between the variables were evaluated using contingency tables and the χ² or Fisher’s exact test. When metastatic group, tumor size and grade were the dependent variables, the univariate and multivariate associations of dependent variables and the prognostic factors, p53 and Ki-67, were analyzed using logistic regression analysis (30). As the ordinal-type dependent variables consisted of more than two categories, the cumulative logistic models (proportional odds model) were used instead of the traditional binary logistic regression analysis. The results of logistic regression were quantified by calculating odds ratios (OR) and cumulative odds ratios (COR), with 95% confidence intervals (95% CI). Kaplan-Meier survival curves for p53 and Ki-67 were calculated for patients with pm or lm and the curves were compared using the log-rank test. Additionally, for the patients with lm, prognostic values of the clinicopathological variables and biomarkers, p53 and Ki-67, for metastases-free and overall survival were analyzed using the Cox proportional hazards model. In all tests, p-values less than 0.05 were considered statistically significant. Statistical calculations were performed using SAS System for Windows, release 8.02/2001 (SAS Institute, Cary, NC).

Results

Associations between T-stage, grade, Heidelberg classification and occurrence of metastases. A significant association between T-stage and the metastatic groups was observed (p<0.001, Table I), indicating that the patients with high T-stage were more likely to develop metastatic cancer. A significant association was also observed between the Heidelberg classification and metastatic groups (p=0.006, Table I), indicating that unclassified type RCC had a high probability of metastases. Six patients had unclassified type RCC, four of whom had sarcomatoid changes, two in the pm group and two in the lm group.

An association between the Heidelberg classification (conventional type versus papillary and chromophobe types)
and grade was observed \((p=0.003)\), with grades being higher in papillary and chromophobe types compared to conventional type. Neither the association between the Heidelberg classification and T-stage (data not shown), nor between grade and metastatic category was statistically significant.

**Comparison of prognostic values of p53 and Ki-67 for T-stage, grade and occurrence of metastases in conventional RCC patients.** p53 protein expression was detected in 15 out of 101 tumors (15%) and Ki-67 protein expression in 21 out of 101 (21%) tumors. The representative positive stainings are presented in Figures 1B and 1D. An association between Ki-67 and p53 expressions \((p<0.001)\); Ki-67 expression was detected in 57.9% of p53-positive and in 14.4% of p53-negative tumors.

Multivariate logistic regression analysis was used to investigate the association of the two biomarkers, p53 or Ki-67, with T-stage, tumor grade and metastases development (Table II). Ki-67 positivity was more common in patients with high T-stage \((p=0.024, \text{Table IIA})\) and this association was also significant in multivariate analysis \((p=0.036, \text{Table IIA})\). p53 positivity was more common in patients with high tumor grade than in those with low tumor grade \((p=0.037, \text{Table IIB})\), but in multivariate analysis this association was not statistically significant. p53 and Ki-67 protein expressions were associated significantly with the metastatic groups \((p=0.020 \text{ and } p<0.001, \text{respectively, Table IIC})\): p53 and Ki-67 were more common in the lm group than in the nm group and more common in the pm group than in the lm group. In multivariate analysis, only Ki-67 protein expression was associated with the development of metastases \((p=0.007, \text{Table IIC})\), indicating that Ki-67 had a stronger association than p53 with the development of metastases. Co-expression of p53/Ki-67 was more common in the pm patients than in the lm patients and none of the nm patients showed double positive staining for p53 and Ki-67 \((p=0.001, \text{Table I})\).

No significant associations were observed between p53 or Ki-67 and Heidelberg classification, age, gender, WHO.
Figure 1. Expression of (A) p53 protein with negative staining, (B) p53 protein with positive staining of 50%, (C) Ki-67 protein with negative staining and (D) Ki-67 protein with positive staining of 15% in renal cell carcinoma tissue. Original magnification x250.
performance status at nephrectomy, number or site of metastases, or response to interferon-α (data not shown).

Prognostic factors for survival. Both p53 and Ki-67 were markers for overall survival in the pm/lm patients (Figures 2 A and B). The median overall survival was 24 months in p53-positive and 59 months in p53-negative patients (log-rank test, \( p=0.030 \)) and 24 months in Ki-67-positive and 63 months in Ki-67-negative patients (log-rank test, \( p=0.031 \)).

Metastasis-free and overall survival from nephrectomy in the group of lm patients is presented in Table III. High T-stage (T3,T4) was associated with shorter metastasis-free survival in univariate analysis (\( p=0.034 \)); these patients had twice the risk of metastatic disease than patients with low T-stage (T1,T2). T-stage was also associated with overall survival (\( p=0.006 \)); patients with high T-stage (T3,T4) had an over three times higher risk of death than patients with low T-stage (T1,T2). Patients with grade 3 tumors had an over three times higher risk of metastatic disease and death when compared to patients with grade 1 (\( p=0.043 \) and \( p=0.054 \), respectively). When all the three grades (1, 2, 3) were included, grade was not significant for metastasis-free and overall survival (univariate analysis), nor was p53 associated with overall survival in the subgroup of lm patients (\( p=0.066 \)). In the lm patients, neither p53, Ki-67, nor Heidelberg classification was associated with metastasis-

Table II. The associations of T-stage, tumor grade, and metastatic category between p53 and Ki-67 protein expressions in conventional RCC (n=101).

<table>
<thead>
<tr>
<th>A. T-stage</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>Univariate analysis</th>
<th>Multivariate analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>p-value</td>
<td>COR (95% CI)</td>
</tr>
<tr>
<td>p53 total No</td>
<td>39</td>
<td>24</td>
<td>29</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>5 (13)</td>
<td>3 (13)</td>
<td>4 (14)</td>
<td>2 (40)</td>
<td>0.409</td>
<td>1.5 (0.6 to 4.4)</td>
</tr>
<tr>
<td>Ki-67 total No</td>
<td>39</td>
<td>24</td>
<td>29</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>4 (10)</td>
<td>6 (25)</td>
<td>7 (24)</td>
<td>3 (60)</td>
<td>0.024</td>
<td>2.9 (1.1 to 7.3)</td>
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</table>

<table>
<thead>
<tr>
<th>B. Tumour grade</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>Univariate analysis</th>
<th>Multivariate analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>p-value</td>
<td>COR (95% CI)</td>
</tr>
<tr>
<td>p53 total No</td>
<td>34</td>
<td>48</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>3 (9)</td>
<td>6 (13)</td>
<td>6 (32)</td>
<td>0.037</td>
<td>3.1 (1.1 to 8.9)</td>
</tr>
<tr>
<td>Ki-67 total No</td>
<td>34</td>
<td>48</td>
<td>19</td>
<td></td>
<td></td>
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<tr>
<td>positive</td>
<td>4 (12)</td>
<td>11 (23)</td>
<td>6 (32)</td>
<td>0.080</td>
<td>2.3 (0.9 to 5.7)</td>
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<table>
<thead>
<tr>
<th>C. Metastatic category</th>
<th>nm</th>
<th>lm</th>
<th>pm</th>
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<th>Multivariate analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>p-value</td>
<td>COR (95% CI)</td>
</tr>
<tr>
<td>p53 total No</td>
<td>46</td>
<td>33</td>
<td>22</td>
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<td></td>
</tr>
<tr>
<td>positive</td>
<td>4 (9)</td>
<td>4 (12)</td>
<td>7 (32)</td>
<td>0.020</td>
<td>3.4 (1.2 to 9.7)</td>
</tr>
<tr>
<td>Ki-67 total No</td>
<td>46</td>
<td>33</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>4 (9)</td>
<td>7 (21)</td>
<td>10 (45)</td>
<td>&lt;0.001</td>
<td>5.0 (1.9 to 12.7)</td>
</tr>
</tbody>
</table>

p53 and Ki-67 were explanatory variables. T-stage, tumor grade and metastatic category were dependent variables in cumulative logistic regression analysis. COR=cumulative odds ratio. In multivariate analysis* both explanatory variables in the same model. Four patients were not evaluable for T-stage. nm=no metastases during at least 7.5 years’ observation, lm=later developed metastatic disease (time to metastases > 0 months), pm=metastasised disease at presentation (time to metastases = 0 months).
free survival and, in addition, neither Ki-67 nor the Heidelberg classification was associated with overall survival. There was no association between gender or age at nephrectomy and metastasis-free or overall survival.

Discussion

Tumor stage and grade have previously been identified as the most important prognostic factors in RCC (12, 14). RCC is a heterogeneous disease, and currently its natural course is still unpredictable, e.g., the prognosis of patients at a similar stage of locally confined RCC, varies greatly (9).

Biomarkers, like p53 and Ki-67, are candidates for defining prognostic subgroups (14, 20), and are also, as shown in the present study, of value in predicting survival in metastatic disease. RCC is characterized by high resistance to radiation and chemotherapy (3-5), which may, in addition to overexpression of the MDR-1 gene (31), be due to the suppression of apoptotic mechanisms for cellular response to stresses, such as the p53 tumor suppressor pathway (32).

P53-mediated targeted therapies - possible future treatments - such as gene therapy (33) and PRIMA-1 (a drug for p53 reactivation and induction of massive apoptosis) (34), are currently under investigation. The aim of the present study was to investigate the incidence of p53 and Ki-67 in primary
RCC tumors at different stages according to occurrence of metastases and the impact of these biomarkers on the survival of RCC patients. The size and distribution of the patient material in the study was typical for RCC studies. The analyses were performed in not only all the collected patients, but also in those with conventional type RCC, to achieve a more homogenous group.

The incidence of p53- and Ki-67-positive expression in RCC tumors was low but similar to that in other RCC studies (35-37). It is known that in addition to melanoma, RCC belongs to the type of tumors with a low incidence of p53 mutations compared to, e.g., prostate and bladder cancer (38). Low p53 mutation in different cancers (38) and low immunohistochemical staining of RCC tissue blocks for the p53 protein in this and other studies (35, 37) suggest that mutations in p53 result in an accumulation of the p53 protein. The association between p53 and Ki-67 protein expressions in the present study was in accordance with findings in other studies (29, 37), indicating that p53 accumulation and increased cell proliferative activity are parallel phenomena in RCC.

The association between p53 and tumor grade in the present study was weak (only in univariate analysis). Nor was such an association observed in a recent microarray study (39). In both studies, the nuclear grade was determined according to the WHO guidelines. Our results and others (37, 40) show an association between Ki-67 and high T-stage and metastasis development, indicating Ki-67 as a marker for aggressive disease in RCC with an increased risk of early metastasis development. The present study also demonstrated an association between the Heidelberg classification and metastasis development, indicating that the unclassified tumor type metastasizes with high probability. No association was observed between tumor grade and metastasis development when considering all the patients or only those who later developed metastases.

In the present study, Ki-67 was a stronger prognostic marker for the development of metastases than p53. However, a combination of prognostic markers may better specify prognostic subgroups than observation of a single marker, as shown in a recent study, where p53 and mdm2, a negative regulator of p53 (35), showed stronger association with poor survival. In that study, p53 alone was not an independent predictor of survival. The present study showed that double positivity for p53 and Ki-67 expression in RCC patients seemed to indicate a higher probability of metastases than single markers. Thus, combining p53 and Ki-67 increases their ability to predict the development of metastases.

Earlier published results on the associations of p53 protein expression with survival have been controversial; some studies have suggested that positive p53 protein expression associates with poor survival (41, 42), while others have observed no association between p53 and survival (35, 37). In a tissue array study on metastasized patients, overexpression of p53 was associated with impaired disease-specific survival in renal carcinoma (43). However, in the present study, no association between p53 and survival was observed. The difference between the previous and the present study was in the classification of metastases: Kim and coworkers (43) classified both distant and local lymph node metastases as metastatic disease, whereas in the present study only tumors with distant metastases were classified as metastatic.

This study also indicated that p53 and Ki-67 were not able to predict which patients would develop metastatic disease after nephrectomy. The finding on the association between Ki-67 and survival after nephrectomy varies in different studies (40, 44, 45). Interestingly, the present findings suggest that p53 and Ki-67 predict poor survival in RCC patients with metastatic disease and can thus help in determining metastatic patients with a poor prognosis and those who would benefit from aggressive treatment, such as high-dose interleukin-2 (7). This is beneficial because metastatic RCC is an extremely heterogeneous disease, with patients having an overall survival from a few months to several years, and to date, no biomarkers in routine use are capable of predicting the survival of patients with RCC metastases.

A uniform staging classification, the TNM staging system, has improved the division of patients into radical or partial nephrectomy cases (3, 46), and additionally, increased the co-operation between oncologists and pathologists concerning the outcome of RCC patients (46). The 1997 TNM classification increased the cut-off between T1 and T2 tumors from 2.5 cm to 7 cm, to increase the difference in the survival of these two tumor types (16). The TNM classification was last revised in 2002, when T1 was divided into T1a and T1b by a cut-off point of 4 cm according to suitability for partial nephrectomy (17). In this study, T-stage was found to be a prognostic factor for metastasis-free and overall survival in RCC patients, who later developed metastatic disease: patients with high T-stage had twice the risk of metastatic disease and three times the risk of death compared to patients with low T-stage, indicating that as the tumor size increases the more aggressive its growth becomes. These results parallel a previous observation (36), and confirm recent analyses on the predictive power of T-stage in the 1997 pTNM classification (9, 46).

In the present study, T-stage was found to be an important factor in predicting the survival of patients who underwent nephrectomy. Therefore, T-stage can be used in estimating the correct duration and frequency of surveillance of RCC patients after nephrectomy. Additionally, high T-stage has been used as an inclusion criterion for adjuvant treatments in trials (10, 11). Previously, it has been suggested that T-stage is not an important prognostic factor in the
survival of patients who have neither lymph node nor distant metastases (47). However, the therapeutic value of lymph node dissection remains unproven (48). For this reason, extensive lymph node dissection was not carried out; and no data on metastatic lymph nodes in nephrectomized patients were available in the present study. Our results suggest that T-stage alone is a valuable factor for survival, even when the status of lymph nodes is unknown.

Finally, in the group of patients with later metastases, tumor grade was not associated with overall survival. Several other studies have also failed to demonstrate any difference in the survival of patients with different grades (37, 41, 49). This is partly because, as yet, no consensus has been reached on a universal tumor grading system (15). However, the present results did point out differences in metastasis-free survival between the highest and the lowest grades, even though when all three grades were included, the differences were no longer statistically significant.

In conclusion, specification of the roles of tumor-related molecular prognostic factors can improve our understanding of the prognosis and planning of treatment strategies in RCC. Molecular staging could be a tool for more individualized treatment than is currently available. The p53 and Ki-67 protein expressions reflect an aggressive disease phenotype with rapid tumor growth and early dissemination in RCC. It remains to be shown whether p53 and Ki-67 will be valuable for selecting patients for new adjuvant treatment programs.

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References

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