mdm2 Expression as a Prognostic Indicator in Clear Cell Renal Cell Carcinoma: Comparison with p53 Overexpression and Clinicopathological Parameters

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ABSTRACT
The present study was designed to analyze the expression of p53 and mdm2 in clear cell renal cell carcinoma with special emphasis on their association with tumor grade and clinical outcome. In particular, the value of individual protein overexpression as well as combined p53/mdm2 positivity was evaluated because both proteins are functionally connected, and their expression is controlled by an autoregulatory feedback loop. A cohort of 97 clear cell renal cell carcinomas was analyzed. The overexpression of mdm2 and p53 proteins was investigated on paraffin-embedded material by using monoclonal antibodies. Eighteen tumors showed mdm2 positivity, whereas 35 of the tumors overexpressed p53. Whereas p53 and mdm2 positivity correlated significantly ($P = 0.00004$), no correlation could be found between mdm2 protein overexpression and tumor stage, lymph node involvement, and presence of distant metastases. Mdm2 positivity was found significantly more frequently in tumors of higher grade. In univariate analysis, there was a statistically significant correlation between p53 and mdm2 overexpression in the same tumor and poor survival ($P = 0.000179$). Multivariate analysis revealed that coincident mdm2/p53 co-overexpression, the presence of distant metastases, and tumor grade were independent predictors for tumor progression. Our results indicate that mdm2/p53 co-overexpression, nuclear grade, and preoperative presence of distant metastasis are independent predictors for poor survival.

INTRODUCTION
The TP53 gene is located on the short arm of chromosome 17 (17p13.1) that encodes the nuclear protein p53, whereas the MDM2 gene maps to the long arm of chromosome 12 (12q13–14) and encodes mdm2 protein (1). p53 protein is involved in cell cycle control but can be inactivated by binding to mdm2 or viral proteins. Mdm2 is a cellular proto-oncogene product that inhibits the transcriptional transactivation activity of p53 and therefore acts as a negative regulator for the tumor suppression function of p53. The p53 protein regulates the mdm2 gene at the level of transcription by an intronic promoter. This creates a feedback loop that regulates both the activity of p53 protein and the expression of the mdm2 gene (2, 3). The present study was undertaken to find out whether there is a correlation between mdm2 and p53 overexpression and the prognostic value of mdm2 positivity with or without p53 protein overexpression.

MATERIALS AND METHODS
Specimens. Of 194 nephrectomy specimens resected at the Department of Urology, University of Vienna, between 1981 and 1988, 120 tumors were classified as clear cell RCC.2 Adequate clinical follow-up, which included laboratory values (complete blood cell count, platelets, erythrocyte sedimentation rate, creatinine, electrolytes, alkaline phosphatase, γ-glutamyltransferase, and urine), abdominal ultrasound, chest film every 3 months, and computed tomography or magnetic resonance imaging once a year, was available for 97 clear cell RCCs. Follow-up ranged from 0.4–175.2 months, with a mean follow-up interval of 49.2 months (median, 38.4 months). There were 36 (37%) female and 61 (63%) male patients, ranging in age from 38–83 years (mean age, 60 years). The Fuhrman system of nuclear grading (4) and the International Union Against Cancer tumor-node-metastasis (TNM) system for tumor staging (5) were used (Table 1). Thirteen patients showed only lymph node metastases, four patients showed only distant metastases, and two patients showed lymph node and distant metastases at the date of operation. One block representing the highest histological grade was chosen from each tumor and prepared for immunostaining.

Immunostaining. Sections (2 μm thick) were deparaffinized and pretreated in a microwave oven (20 min, 120 W; 3 × 5 min, 450 W) in citrate buffer [0.01 M (pH 6)]. For detection of mdm2 protein, a monoclonal antibody against MDM2 (Ab-1; clone IF2; Oncogene Science, Uniondale, NY; dilution, 1:50) was used with an overnight incubation at 4°C. p53 protein was detected using monoclonal antibody DO-1 (Immunotech, Marseille, France; dilution, 1:20) with 60 min of incubation at room temperature. The avidin-biotin complex method (Vectastain Standard ABC Kits; Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine tetrachloride were used for visualization of the signal in the case of p53, and the APAAP Kit (DAKO Corp., Carpinteria, CA) and Fast Red (DAKO Corp.) were used for visualization of the signal in the case of mdm2. The nuclei were finally counterstained slightly with hematoxylin. Sections

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2 The abbreviation used is: RCC, renal cell carcinoma.
treated without primary antibodies served as negative controls, and colon cancer sections were used as positive controls. Only nuclear staining was rated positive. We counted at least 500 cells within a hot spot using an integration grid. Because mdm2

\[ \text{cells (Fig. 1) never represented more than 10\% (6)} \]

cells, we chose a 1\% threshold; in the case of p53, a focal staining of 5\% nuclei was considered positive (7) to rule out single positive cells (8).

**Statistical Analysis.** Pearson’s \( \chi^2 \) test was used to evaluate the interrelations between mdm2 positivity and p53 overexpression, histological grade, tumor stage, lymph node involvement, and metastatic spread. Disease-free survival curves of the RCC patients were estimated according to the Kaplan-Meier method. Statistical analyses of the differences between curves were performed using the log-rank test. Variables that significantly influenced survival (\( P < 0.05 \)) in the univariate analysis were entered into a multivariate Cox regression model. In all of the analyses, the significance level was set at 0.05.

**RESULTS**

**Expression of mdm2 and p53 Protein**

Positive staining for mdm2 was detected in 5 of 44 grade 2 clear cell RCCs (11.36\%), 9 of 27 grade 3 tumors (33.33\%), and 4 of 18 grade 4 carcinomas (22.22\%). None of eight grade 1 tumors showed mdm2 positivity. mdm2 reactivity was seen statistically more frequently in high-grade RCC (grades 3 and 4) than in low-grade RCC (grades 1 and 2; \( P = 0.01490 \)). In contrast to this T category, lymph node involvement and metastatic spread did not correlate with mdm2 expression. p53 positivity was observed in 35 of 97 (36\%) tumors. p53 overexpression was more often found in high-grade tumors (\( P = 0.01456 \)) but was not related to tumor stage or the presence of distant metastasis. Lymph node involvement and mdm2 positivity were significantly associated with p53 protein overexpression (\( P = 0.04001 \); \( P = 0.00004 \); Table 2).

**Survival Analysis in Clear Cell Carcinoma**

**Univariate Analysis.** Patients with high-grade tumors had a significantly shorter disease-free survival time than those with grade 1 and grade 2 tumors (\( P = 0.00004 \); Fig. 2), whereas tumor stage did not give prognostic information. Lymph node involvement (\( P = 0.02703 \)) and preoperative metastatic spread (\( P = 0.00427 \)) are significant prognostic markers. Positive immunostaining for mdm2 was strongly associated with tumor progression (\( P = 0.00113 \); Fig. 3). Similarly, we found a strong correlation between absence of p53 overexpression and progression-free survival (\( P = 0.00291 \); Fig. 4). When comparing the combined phenotypes for protein overexpression of mdm2 and p53 (group A, mdm2−/p53−; group B, mdm2+/p53− and mdm2−/p53+; and group C, mdm2+/p53+), an excellent correlation was observed between positive phenotype and poor prognosis.

### Table 1 Distribution of histological grade and stage

<table>
<thead>
<tr>
<th>Grade</th>
<th>pT1</th>
<th>pT2</th>
<th>pT3A</th>
<th>pT3B</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>8</td>
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<td>2</td>
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<tr>
<td></td>
<td>6</td>
<td>24</td>
<td>40</td>
<td>27</td>
<td>97</td>
</tr>
</tbody>
</table>

### Table 2 Relationship between mdm2 positivity and p53 positivity

\( (\chi^2 = 16.66; P = 0.00004) \)

<table>
<thead>
<tr>
<th>mdm2−</th>
<th>mdm2+</th>
<th>Totals</th>
<th>% mdm2 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53−</td>
<td>58</td>
<td>4</td>
<td>62</td>
</tr>
<tr>
<td>p53+</td>
<td>21</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>18</td>
<td>97</td>
</tr>
</tbody>
</table>

**Fig. 1** Immunohistochemical staining for mdm2 protein in a grade 2 clear cell RCC. Original magnification, ×400.
Comparing group B with the other categories, a significant difference was observed with group A \((P = 0.04)\) as well as with group C \((P = 0.03)\).

**Multivariate Analysis.** In multivariate analysis including histological grade, tumor stage, lymph node and distant metastases, mdm2 positivity, and p53 overexpression, only metastatic spread and tumor grade remained statistically significant prognostic markers (Table 3). Because of the strong functional relation between mdm2 and p53, we entered the value p53\(^+1\)/mdm2\(^+1\) versus others in the second multivariate model. The coincident mdm2/p53 positivity became an additional relevant predictor of progression-free survival (Table 4). Moreover the \(P\) of the variable mdm2/p53 in the second analysis is smaller than \(\alpha\)/2. This leads to a significant result for this variable even if the Bonferroni method is used to correct for multiplicity.

**DISCUSSION**

The best predictors of behavior in RCC have been tumor stage and histological grade (4, 9, 10, 11). Because RCC is characterized by a highly unpredictable clinical outcome, additional independent prognostic indicators should be sought. p53 protein overexpression is associated with an aggressive biological behavior (8). The p53 protein and mdm2 are functionally closely related. The transcription of the mdm2 gene is regulated...
by wild-type p53 protein (2, 3). In addition, the mdm2 proto-oncogene product has been shown to bind to p53 and inactivate its physiological role as a transcription and cell cycle regulator (12) and also to inhibit p53 function on mdm2 gene transcription. Many tumors show mdm2 overexpression without mdm2 amplification (13, 14). Neither Imai et al. (15) nor Moch et al. Besides gene amplification, other mechanisms not yet known—yet responsible for the mdm2 overexpression—have to exist. The impact of gene amplification on clinical outcome in combination with p53 overexpression is of interest.

This study has emphasized the value of mdm2 in selecting a poor prognosis group within a cohort of patients with clear cell RCC. Of 97 clear cell RCCs examined, 18 (19%) showed immunohistochemical staining for mdm2. The percentage of mdm2+ tumors is somewhat lower than that seen in the results of Moch et al. (6), a difference that may be caused by the fact that we used a cutoff level of 1% positive nuclei. p53 positivity could be found in 35 of 97 (36%) RCCs, which is comparable with previously published data (8, 17–19) but is nearly twice as high as the results of Moch et al. (6) using a different antibody. We could not find any association between histological grade or tumor stage and mdm2. Immunostaining for mdm2 was associated with p53 overexpression and progression-free survival. When patients were divided into three groups on the basis mdm2 and/or p53 positivity, there was a significant trend for poorer survival in the “double positive” group, similar to find-
mdm2 Expression in Renal Cell Carcinoma

Table 3 Multivariate analysis using Cox proportional hazard regression model including p53 and mdm2 as separate variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>HRa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading (grades 1 and 2 vs. grades 3 and 4)</td>
<td>2.44</td>
<td>0.005</td>
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<tr>
<td>Lymph node metastases (pN0 vs. pN1 and pN2)</td>
<td>1.44</td>
<td>NSb</td>
</tr>
<tr>
<td>Distant metastases (pM0 vs. pM1)</td>
<td>3.51</td>
<td>0.008</td>
</tr>
<tr>
<td>p53</td>
<td>1.34</td>
<td>NS</td>
</tr>
<tr>
<td>mdm2</td>
<td>1.97</td>
<td>NS</td>
</tr>
</tbody>
</table>

*a* HR, hazard ratio.
a NS, not significant.

Table 4 Multivariate analysis using Cox proportional hazard regression model including mdm2/p53 co-overexpression as a variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>HRa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading (grades 1 and 2 vs. grades 3 and 4)</td>
<td>2.41</td>
<td>0.007</td>
</tr>
<tr>
<td>Lymph node metastases (pN0 vs. pN1 and pN2)</td>
<td>1.58</td>
<td>NSb</td>
</tr>
<tr>
<td>Distant metastases (pM0 vs. pM1)</td>
<td>3.94</td>
<td>0.003</td>
</tr>
<tr>
<td>mdm2/p53 vs. mdm2 and/or p53—</td>
<td>2.34</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*a* HR, hazard ratio.
a NS, not significant.

ings in adult soft tissue sarcomas (20). In multivariate analysis including histological grade, tumor stage, lymph node positivity, presence of distant metastases, and mdm2/p53 co-overexpression, the independent predictors of progression-free survival were distant metastasis, coincident mdm2/p53 positivity, and tumor grade. Our data contradict the results of Moch et al. (6), who found that mdm2 overexpression offers no prognostic information in RCC. This difference might be caused by the fact that one-third of our patients showed organ-confined tumors, whereas Moch et al. entered only patients with pT3 tumors in their study. Additionally, in our study, 20% of the patients showed metastases at the date of operation compared with 38% of the patients in the study of Moch et al. (6). For survival analysis, we used progression-free survival time to evaluate the prognostic value of mdm2 and p53, whereas Moch et al. (6) used overall survival for their survival analyses.

In conclusion, this study shows that mdm2 as well as p53 protein overexpression is associated with tumor progression. The significant correlation of mdm2 and p53 positivity and the worse prognosis of patients with tumors showing coexpression of both proteins suggest that mdm2/p53 co-overexpression may be a clinically useful prognostic marker capable of identifying clear cell RCC patients with poor prognosis who may benefit from enhanced adjuvant therapy.

ACKNOWLEDGMENTS

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