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# p53 protein expression but not mdm-2 protein expression is associated with rapid tumor cell proliferation and prognosis in renal cell carcinoma

**Abstract** The clinical course of renal cell carcinoma (RCC) is highly variable. Overexpression of the p53 protein has been suggested as a possible prognostic parameter in RCC. Overexpression of the mdm-2 oncogene product has been shown to interact with the p53 function. To investigate the immunohistochemical overexpression of mdm-2 protein in comparison with that of p53 protein in RCC, 50 nonpapillary pT3 RCCs were immunostained for p53 protein (DO-7) and mdm-2 (IF2). Tumor growth fraction (Ki-67 labeling index; MIB-1) was determined by immunohistochemistry. p53 positivity was detected in 16% of tumors. mdm-2 overexpression was seen in 30% of RCCs. There was a significant association between p53 and mdm-2 immunostaining ($P=0.0006$), suggesting that mdm-2 protein may contribute to p53 protein stabilization in RCC. p53 overexpression was associated with a high Ki-67 LI ($P=0.0002$), suggesting that p53 overexpression is involved in growth control in RCC. Survival analysis showed that Ki-67 LI ($P=0.04$) and p53 overexpression were associated with poor prognosis ($P=0.0021$), whereas mdm-2 overexpression was not related to patient outcome ($P=0.73$). A Cox regression analysis revealed tumor stage ($P<0.001$) and p53 overexpression ($P<0.05$) to be independent prognostic parameters. It is concluded that p53 but not mdm-2 may be of practical relevance in predicting patient prognosis in RCC.

**Key words** Renal cell carcinoma · Prognosis · p53 · mdm-2 · Survival · Proliferation

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## Introduction

Renal cell carcinoma (RCC) accounts for an estimated 20 000 new cases/year in the United States [1]. Five-year survival is about 45% in tumors extending beyond the renal capsule (pT3). The outcome of patients with pT3 tumors cannot be satisfactorily predicted in individual patients. Better knowledge of the individual prognosis would be an aid in selecting patients for adjuvant treatment modalities.

The p53 protein is a product of the most commonly mutated gene in human tumors characterized to date. Mutations of the p53 gene can result in an increased half-life of the p53 protein, making it detectable by immunohistochemistry [2]. However, immunohistochemical p53 overexpression can also be caused by nonmutational mechanisms, and not all mutations result in p53 overexpression [3]. Recent studies have suggested that p53 overexpression is associated with poor prognosis and with sarcomatoid transformation in RCC [4, 5].

Recently, an endogenous gene product has been identified that can bind to p53 protein and affect its function: the mdm-2 (murine double minute-2) protein [6, 7]. The *mdm-2* oncogene on chromosome 12q13–14 encodes a nuclear phosphoprotein that may interact with both mutant and wild-type p53 proteins [7]. The mdm-2 gene product can inhibit p53-mediated transactivation, and it has been suggested that p53 and mdm-2 reciprocally regulate each other [6, 7]. The *mdm-2* gene has been found to be deregulated in human sarcomas, gliomas, breast carcinomas, and leukemias [8–10].

In this study we have used immunohistochemistry to investigate the frequency of mdm-2 protein overexpression in comparison to that of p53 protein overexpression in a series of 50 consecutive pT3 nonpapillary RCCs. To study the biological significance of these parameters, p53 and mdm-2 overexpression were correlated with Ki-67 labeling index (LI) and patient prognosis.

## Materials and methods

### Patients and tumor samples

Tumor samples of 50 consecutive, previously untreated nonpapillary RCCs extending beyond the renal capsule (pT3) were formalin fixed and paraffin embedded. Four-micrometer sections were used for hematoxylin and eosin (H & E) staining and immunostaining. Histologic classification was performed as described by Weiss et al. [11]. The tumors were graded according to the Thoenes grading system [12] and staged according to the UICC classification [13]. Metastatic status at the time of surgery was determined from patient charts. Follow-up and overall survival data of all patients were obtained from the attending physicians.

### Immunohistochemistry

Formalin-fixed tumors were immunostained for p53 and mdm-2 protein. Proliferative activity was assessed by detecting the Ki-67 protein. All immunohistochemical examinations were performed using the avidin-biotin-enhanced immunoperoxidase technique as recently described [14]. A microwave/citrate buffer procedure was employed to improve antigen retrieval as described [14]. Negative control slides were prepared by omitting the primary antibody.

Sections of the paraffin samples were immunostained for mdm-2 protein using the IF-2 monoclonal antibody (IF2; 1:20, Calbiochem, Cambridge, MA). IF2 recognizes an epitope in the amino-terminal portion of the human mdm-2 protein. Positive controls for mdm-2 consisted of metastatic melanoma with known positivity.

The anti-p53 antibody (DO-7, 1:400, Dako, Glostrup, Denmark) was used to detect p53 immunoreactivity as previously described [14–16]. The anti-p53 antibody DO-7 reacts with wild-type and mutant p53 protein. Positive controls for p53 consisted of breast cancer with known positivity. Only nuclear immunoreactivity for the above antibodies were scored as positive. The fraction of cells showing nuclear p53 and mdm-2 positivity was estimated in all cases.

The monoclonal antibody MIB1 (1:800; Dianova, Hamburg, Germany) was used to detect the Ki-67 antigen. The Ki-67 antigen represents a nuclear cell proliferation-associated protein expressed in G1, S, G2, and M phases of cell cycle, but not in nonproliferative G0 cells [17]. Reading of the stained sections was done without knowledge of the clinical data. Ki67 labeling indices (percentage of positive cells, LI) were determined by scoring 300 tumor cells in tumor areas with the highest density of Ki-67-positive cells. Nuclei were considered Ki-67 positive if any nuclear staining was seen. Only nuclear staining was considered. The median percentage of positive cells was used as a cutoff point to define groups with low and high proliferative activity.

### Statistics

Contingency table analysis was used to examine the relationship between p53 immunostaining, mdm-2 immunostaining, tumor grade, and stage. Student's *t*-test was employed to examine the relationships among both p53 and mdm-2 with Ki-67 labeling index (LI). Life tables were estimated by the Kaplan-Meier statistic and survival curves were compared by a log-rank test. Surviving patients were censored at the date of their last clinical control. Cox regression analysis was used to test for independent prognostic significance.

## Results

### Clinicopathologic data of patients

Twenty-seven tumors were grade 2 and 23 tumors were grade 3. The mean tumor diameter was 7.7 ± 2.7 cm (median 7 cm, range 3.5–15 cm). Thirty-one tumors were nonmetastatic (pT3 N0, cM0) and 19 metastatic (pT3, pN1, or cM1) at the time of diagnosis. Tumor size and histologic grade were not associated with metastatic disease at presentation (data not shown). The median follow-up period for the patients was 37 months (range 2–108 months). Thirty-two patients died during the follow-up period. All of the patients who died had metastatic disease at their last clinical control.

### Mdm-2

Mdm-2 staining was always nuclear (Fig. 1). No immunoreactivity was found in normal renal parenchymal cells, stromol cells, or lymphoid cells. In tumors, nuclear mdm-2 overexpression was always heterogeneous, with a few scattered mdm-2-positive cells within the tumor. Mdm-2-positive cells never represented more than 10% of the tumor cells. Mdm-2-positive nuclei were identified in 15 of 50 pT3 RCC cases (30%) studied. There was no association between mdm-2 positivity and tumor diameter, tumor grade, or stage (Table 1). Mdm-2 positivity was not associated with patient prognosis (Fig. 2).

### p53

A nuclear p53 overexpression was observed in 8 of 50 (16%) cases. The p53 immunoreactivity was confined to tumor cell nuclei. A weak p53 cytoplasmic positivity was seen in a few cases. Cytoplasmic positivity was considered negative. Tumors did not stain homogeneously. No case contained more than 10% immunoreactive tumor cells. p53 overexpression was not related to tumor diameter, tumor grade, or metastasis (Table 1). Comparison of p53 overexpression with patient prognosis revealed a significantly better prognosis for p53-negative than for p53-positive tumors ($P = 0.00021$; Fig. 3).

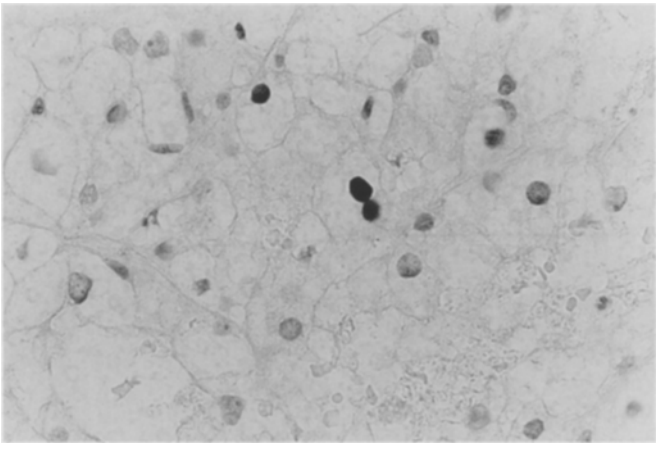

**Fig. 1** Immunostaining of mdm-2. Renal cell carcinoma showing nuclear staining, ×560

**Table 1** p53/mdm-2 overexpression and tumor phenotype in pT3 RCC

| | | *n* | Number p53 positive[a] (%) | *P* value* | Number mdm-2 positive[a] (%) | *P* value* |
|---|---|---|---|---|---|---|
| Tumor diameter | ≤7 cm | 17 | 3 (18) | | 7 (41) | |
| | >7 cm | 33 | 5 (15) | 0.86 | 8 (24) | 0.36 |
| Tumor grade[b] | G2 | 27 | 2 (8) | | 9 (35) | |
| | G3 | 23 | 6 (26) | 0.18 | 6 (26) | 0.74 |
| Metastasis | N0, M0 | 31 | 3 (10) | | 8 (26) | |
| | N1 or M1 | 19 | 5 (26) | 0.25 | 7 (37) | 0.61 |

* Chi-square test
[a] Defined for positivity; see text
[b] According to Thoenes grading system

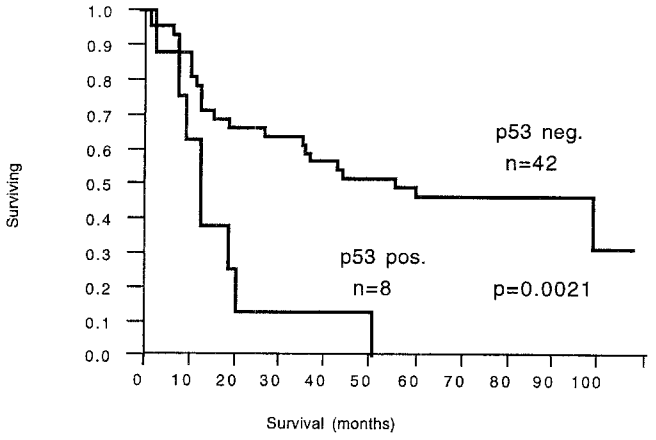


**Fig. 2** Overall survival of patients with pT3 RCC according to p53 overexpression

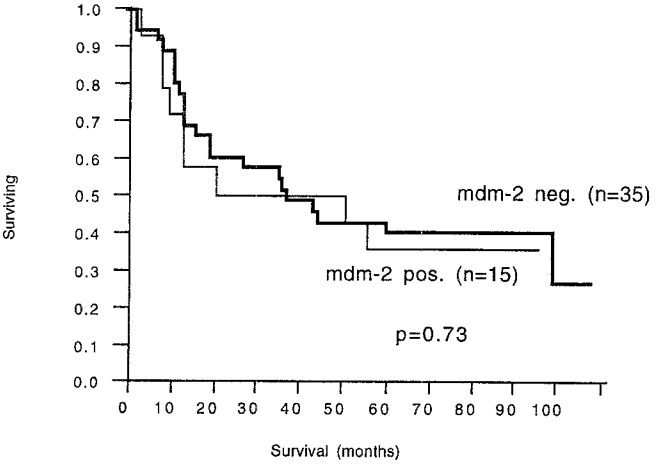


**Fig. 3** Overall survival rates of patients with pT3 RCC according to mdm-2 overexpression

p53 positivity was strongly associated with mdm-2 overexpression. Seven out of 8 p53-positive tumors were mdm-2 positive, but only 8 of 42 p53-negative tumors ($P=0.0006$).

**Table 2** Ki-67 LI and tumor phenotype

| | | *n* | Ki-67 LI | *P* value* |
|---|---|---|---|---|
| Tumor grade[a] | G2 | 27 | 6.0 ± 5.3 | |
| | G3 | 22 | 11.7 ± 10.6 | 0.0184 |
| Metastasis | N0, M0 | 30 | 7.1 ± 8.4 | |
| | N1 or M1 | 19 | 10.9 ± 8.5 | 0.1389 |
| p53[b] | Negative | 41 | 6.7 ± 6.7 | |
| | Positive | 8 | 18.3 ± 10.5 | 0.0002 |
| mdm-2[b] | Negative | 35 | 6.4 ± 7.2 | |
| | Positive | 15 | 13.6 ± 9.4 | 0.0049 |

* Student's t-test
[a] According to Thoenes grading system
[b] Defined for positivity; see text

**Table 3** p53, mdm-2, and Ki67 LI

| | *n* | Ki-67 LI (%) | *P* value* |
|---|---|---|---|
| mdm-2 negative | 35 | 6.4±7.2 | 0.0001[a] |
| mdm-2 positive/p53 negative | 8 | 7.3±4.7 | 0.0017[a] |
| mdm-2 positive/p53 positive | 7 | 20.8±8.3 | |

* Student's *t*-test
[a] Versus mdm-2 positive/p53 positive

## Tumor growth fraction (Ki-67 LI)

Forty-nine tumors stained successfully for Ki-67. One tumor showed Ki-67-negative mitoses and a total lack of Ki-67-positive cells. This tumor was excluded from Ki-67 analysis. The mean Ki-67 LI was 8.4±8.6% (range 0.1–32.4%, median 6%), while normal renal tissue had Ki-67 LIs fewer than 2%. There was a significant relationship between Ki-67 LI and histologic grade ($P=0.02$), but Ki-67 LI was not related to presence of metastases (Table 2).

Both p53 ($P=0.0002$) and mdm-2 ($P=0.005$) were associated with rapid tumor cell proliferation in univariate analysis (Table 2). However, the combined analysis of p53 and mdm-2 revealed that this was driven by p53 positivity (Table 3).

**Table 4** Cox regression analysis

| | Relative risk | *P* |
|---|---|---|
| Metastases | 4.41 | 0.0001 |
| p53 | 3.00 | 0.0275 |
| Grade | 1.21 | 0.5291 |
| Ki-67 | 1.11 | 0.7561 |

### Multiparameter analysis

Cox regression analysis including the variables metastatic disease, histologic grade, p53 positivity, and Ki-67 LI showed that only metastatic tumor disease and p53 overexpression were independent prognostic factors, the relative risk of progression being 4.41 ($P<0.0001$) and 3.0 ($P=0.0275$), respectively. Histologic tumor grade and Ki-67 LI provided no additional prognostic information (Table 4).

## Discussion

Tumor stage and tumor grade are regarded as the major pathomorphologic prognostic factors of survival in RCC [12, 18, 19]. The biological relevance of p53 immunoreactivity in RCC [4, 20] is controversial. In this set of patients with identical tumor stage and histologic tumor type, p53 overexpression by immunohistochemistry was sig-nificantly associated with patient survival, while histologic grade and tumor size yielded no prognostic information.

In this study, immunohistochemistry was used to determine p53 overexpression. A nuclear p53 overexpression was found in 16% of 50 nonpapillary pT3 RCCs. This is similar to the rate found in several other studies (range from 11% to 33%) [4, 21–23]. Overexpression of the p53 protein is often caused by missense mutations of the *p53* gene, since *p53* gene mutations lead to stabilization of the protein which can then be detected by immunohistochemistry. However, several previous studies have shown that mutations of the *p53* tumor suppressor gene are either significantly less frequent than in other tumors [24, 25] or are totally absent in RCC [23]. Therefore, alternative pathways may have caused p53 stabilization in RCC.

p53 stabilization may also be achieved through binding to other cellular proteins, such as the mdm-2 oncoprotein as shown in sarcomas [6, 10]. The significant association of p53 and mdm-2 in our study suggests an interaction between these proteins and raises the possibility that mdm-2 overexpression contributes to p53 protein stabilization in RCC. This could be important in the pathogenesis of these cases, since mdm-2 may deregulate the p53-dependent growth-suppressive pathway [7]. It has been shown that tumor cells can tolerate levels of wild-type p53 that are normally growth-suppressive if they overexpress the *mdm-2* gene [26].

Several mechanisms have been described to induce mdm-2 overexpression including mdm-2 amplification, RNA overexpression, or post-translational stabilization. The *mdm-2* gene is amplified in a significant percentage of human sarcomas [27]. In a recent study by Imai et al. [25], no amplification of the *mdm-2* gene was detected by dot-blot analysis in 49 renal tumors, suggesting that mdm-2 amplification is at least a rare mechanism for mdm-2 overexpression in RCC. The general absence of mdm-2 amplification is also consistent with our previous observation showing a lack of any amplifications in a different set of 42 RCCs by comparative genomic hybridization [28]. Therefore, the high percentage of mdm-2 immunopositivity (30%) suggests that mdm-2 overexpression in RCC could be due to other mechanisms than gene amplifications, such as chromosomal translocations or mutations which could increase the level of mdm-2 protein.

mdm-2 immunohistochemical reactivity in the absence of *mdm-2* gene amplification has been reported in soft tissue sarcomas [29] and on breast carcinomas [30]. Marchetti et al. [30] showed that breast cancer cases with high-level mdm-2 amplifiction had a high percentage (≥10%) of cells with mdm-2 overexpression. In contrast, cases with borderline mdm-2 amplifications or tumors with mdm-2 overexpression in the absence of amplification showed a percentage of mdm-2-immunoreactive cells ranging from 0.1 to 5%. Our finding that in all mdm-2-immunopositive cases the number of immunoreactive tumor cells was low (<10% of tumor cells) is consistent with an overexpression of mdm-2 in the absence of high-level gene amplification in RCC. mdm-2 protein immunoreactivity was not observed in normal epithelial and stromal cells, similar to most other normal tissue examined for mdm-2 [31]. mdm-2 immunoreactivity in normal tissue was only reported by Wiethege et al. [32] in bronchial epithelial cells.

A number of previous studies using flow cytometry, mitotic index, proliferating-cell nuclear antigen (PCNA), and Ki-67 immunohistochemistry [33–37] have suggested a prognostic importance of tumor cell proliferation in RCC. Previous studies have examined the relationship between p53 overexpression and tumor proliferation in a variety of tumors including breast, colon, prostate, and lung. Most reports showed an increased tumor proliferation in p53-positive tumors. In the present study a correlation between p53 immunostaining and Ki-67 LI was observed, providing additional evidence that p53 alterations, although rare in RCC, confer a highly aggressive phenotype to these tumors. The association between a high Ki-67 LI and mdm-2 positivity in our study could suggest that mdm-2 overexpression is also involved in promoting RCC tumor cell proliferation. However, the combined analysis of both p53 and mdm-2 overexpression showed that this was driven by p53 overexpression alone. A possible explanation is that mdm-2 overexpression alone is not sufficient to increase tumor aggressiveness. Although p53 overexpression was associated with mdm-2 overexpression, the lack of an association between mdm-2 overexpression and patient prognosis further indicates that immunohistochem-

ical detection of mdm-2 is of little practical relevance in RCC.

Altered p53 overexpression has been suggested to predict adverse prognosis in RCC [4], although these findings have not been consistently confirmed [21, 22]. The analysis of our clinical data showed that p53 overexpression and Ki-67 LI were significantly associated with prognosis if uniparameter analysis was performed. Multiparameter analysis by the Cox hazard model showed that only presence of metastases and p53 overexpression were independent predictors of tumor progression, whereas histologic grade, Ki-67 LI, and mdm-2 overexpression provided no additional prognostic information. This finding emphasizes the potential prognostic usefulness of p53 immunohistochemistry in RCC.

In summary, this study shows that p53 is associated with tumor progression in pT3 RCC. The association between p53 and mdm-2 overexpression suggests that mdm-2 protein overexpression may be involved in the regulation of the p53 protein function in RCC. mdm-2 overexpression without p53 protein overexpression is not associated with tumor cell proliferation and mdm-2 overexpression offers no prognostic information in patients treated by nephrectomy.

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