Impact of p53, MIB-1 and PECAM-1 expression on the prognosis of urothelial carcinoma of the renal pelvis

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Abstract
Objective: Determining whether the overexpression of p53, MIB-1 and PECAM-1 of protein levels is of interest in predicting the prognosis of transitional cell carcinoma of the upper urinary tract (TCC-UUT) with the primary seat in the renal pelvis.

Materials and methods: A univariate and multivariate analysis was conducted for prognosis prediction in a series of 82 patients with TCC-UUT of the renal pelvis who had no metastases at diagnosis (N0/Nx M0) and were treated exclusively with nephroureterectomy. We assessed clinicopathological parameters (age, gender, tumor grade and extent, histological variety, growth pattern, vascular invasion, infiltration of the renal parenchyma, and tumor necrosis) and the immunohistochemical expression of p53, MIB-1 (ki-67) and PECAM-1 (CD31) in sections performed with tissue microarray (TMA).

Results: A total of 47.6% of the patients had high-grade lesions according to the USIP-WHO classification. The growth pattern was flat in 15.85%. The distribution by T category was: 3.7% pTa, 51.2% pT1, 11% pT2, 29.3% pT3 and 4.9% pT4. The mean follow-up was 46.8 ± 38.5 (range 4–172) months. The median survival was reached at 57 (95% CI 44–63) months. The univariate analysis revealed that survival in these patients is associated with tumor size (p = .028), histological variety (p < .0001), growth pattern (p < .0001), grade (p < .0001), pT (p = .01), vascular invasion (p = .025), necrosis (p = .004) and overexpression of p53 (p = .0006), PECAM-1 (p = .0036) and MIB-1 (p = .0038). The Cox regression model showed that high-grade (HR, 4.2; 95% CI 1.28–13.79; p = .018), flat-growth pattern (HR, 2.52; 95% CI 1.05–6.03; p = .038) and p53 overexpression (HR, 2.8; 95% CI 1.22–6.44; p = .015) were independent predictors.

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Introduction

Transitional cell carcinoma of the upper urinary tract (TCC-UUT) in the renal pelvis is a rare tumor, with an estimated incidence in the western world of around 2 for every 100,000 people/year.\(^1\) It accounts for 8% of renal tumors and 2–5% of all TCCs.\(^2,^3\) As in any kind of these neoplasms, the main variables defining prognosis are grade and tumor stage.\(^4,^5\) However, the prognosis for patients with morphologically similar tumors usually differs. The standard treatment is radical nephroureterectomy. Nonetheless, one out of every 4 patients dies as a result of the disease within 5 years.\(^6\)

A better knowledge of the individual malignant potential of these kind of neoplasms would provide more rationality when choosing treatment and, possibly, would enable us to achieve better survival rates, even with less comorbidity. We recognize the need to identify new and better prognostic markers in order to be able to establish risk-adjusted therapeutic schemes. Many articles try to establish the role of molecular elements related with cell proliferation, apoptosis, and angiogenesis for prognostic identification in the case of this neoplasm.\(^7,^10\)

The p53 gene is a suppressor gene that modulates cell growth and plays a key role in the response to cell damage and stoppage of cell cycle. When this gene undergoes mutations and loses its function, DNA damage is accumulated, which facilitates the occurrence of urothelial carcinogenesis.\(^11,^14\) Many studies have focused on elucidating the role of this oncoprotein in the renal pelvis, with mixed results.\(^1,^9,^15–18\)

The MiB-1 proliferation index, also known as ki-67, is a cell proliferation marker. It is a nuclear protein complex expressed in the various phases of the cell cycle of proliferating cells (G1, S and G2-M), but not in the G0 phase.\(^13,^20\) Overexpression of MiB-1 is considered a biomarker in TCC of the urinary bladder which predicts the risk of recurrence and death due to cancer after radical cystectomy.\(^1,^22\)

The platelet-endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, is a 140-kda type I integral membrane glycoprotein which is expressed in early and mature endothelial cells, in platelets, in most leukocyte subpopulations, and in some macrophage precursors (dendritic cells) of the bone marrow.\(^2,^24\) It regulates leukocyte migration through the venule walls and plays a decisive role in normal and pathological angiogenesis,\(^18,\) and in integrin activation by forming a complex with the SHP-2 protein tyrosine phosphatase.\(^24\)
We prospectively evaluated the usefulness of p53, Ki-67 and CD31 in the identification of the prognosis of TCC of the renal pelvis treated exclusively with surgery.

Materials and methods

Patients and samples

Prospective study carried out on a series of patients with histological diagnosis of TCC of the renal pelvis without metastasis (NO/Nx M0) treated with radical nephroureterectomy between January 2000 and December 2008. All the registers were included in a database with the approval of the Institutional Review Board. Surgery was performed through an open or laparoscopic approach and lymphadenectomy was carried out according to the surgeon’s preference, without any predefined template. Patients were followed up every 3–4 months in the first year, then every 6 months within the next 4 years, and subsequently on an annual basis in order to rule out recurrence in the retroperitoneum and/or distant metastasis. Follow-up of the urothelial tract was also carried out, although the occurrence of relapses in the bladder was not analyzed in this study. In all patients, TCC was the primary start of the disease, excluding those patients with a history of previous bladder carcinoma. All patients were followed up until their death due to the disease or until the data were censored.

A single pathologist (JIL) evaluated all the specimens, collecting the histological type, histological grade (WHO 1973 USIP/WHO 2004), tumor stage (AJCC/TNM 2010), growth pattern (papillary vs. sessile), vasculolymphatic invasion, renal parenchyma infiltration, and tumor necrosis.

Tissue microarray construction

Tissue microarray (TMA) was performed using tissue samples embedded in paraffin and fixed in formalin from 82 patients. We revised the original preparations, and those containing abundant tumor tissue and those representative of the case were marked with colored ink. For each case, 3 tumor cylinders (0.6 cm in diameter) of the selected areas were transferred to the recipient block. 5-μm sections were prepared from TMA blocks, and stained with hematoxylin–eosin verifying the proper construction of the blocks and that all cases were representative ones. The definitive analysis included 246 tumor cylinders which were fully examined.

Immunohistochemical staining

An immunohistochemical (IHC) study was carried out for p53, MIB-1, and PECAM-1 on 5-μm histological cuts from the TMA described above. Preparations were deparaffinized, rehydrated, and IHC was performed using the streptavidin-biotin-peroxidase method modified with dianobenzidine as chromogen. The panel of antibodies used included p53 (monoclonal, clone DO-7; DAKO, Glostrup, Denmark), MIB-1 (monoclonal, 1/50; DAKO, Glostrup, Denmark), and CD-31 (monoclonal, 1/10; Quartett GmbH, Germany). Positive controls were done with bladder TCC samples which overexpressed antibodies. Negative controls were also done with no antibody application. Sections were revised by the same pathologist.

At least 150 malignant cells were examined for each case. P53 overexpression was defined by the presence of positive nuclear staining in ≥10% of the nuclei. Positive nuclear staining for MIB-1 was evaluated in percentage terms, thus defining the Ki-67 proliferation index for each case. Interquartile ranges defined as sections (0–5%, 6–12%, 13–25%, >25%) were established. For the survival analysis, overexpression of MIB-1 was defined as positive staining in ≥6% of the evaluated nuclei. PECAM-1 was considered to be overexpressed when neoformed vessels were found in tumor thickness, a vessel being identified as any discrete grouping of cells with IHC staining for CD31 at ×200 magnification.

Statistical analysis

A descriptive study and a survival analysis were carried out. The association between variables was studied using the chi-square and Fisher’s tests. The risk of death due to the disease was estimated (cancer-specific mortality) using a univariate analysis, evaluating the survival function according to the Kaplan–Meier method and the log-rank test for the different variables studied. A multivariate analysis was then performed using Cox’s proportional hazards regression model. The estimated hazard ratios (HR) were thus defined with their respective 95% confidence intervals for each variable analyzed, detecting those which independently predicted survival in this model.

Results

The study was carried out on 82 patients. The proportion men:women was 3.8:1. The average age was 66.9 ± 10.6 (34–89) years, with no significant differences being detected between the ages of both sexes (men 66.5 ± 10.3 and women 68.3 ± 11.9; p = 0.52). The mean size of the lesion, according to the information from the histopathological study, was 34.4 ± 18.6 mm (8–120) for the largest diameter. Table 1 shows the distribution of the series taking into consideration the main clinicopathological features. In 26 cases (31.7%) the lesion also affected the ureter, either because they were multifocal lesions (12 cases) or because they showed in situ carcinoma (14 cases). The growth pattern was papillary in 84.2% of the cases and nodular in 15.8%. All the cases were TCC, though 8 (10.25%) showed mixed histology composed of high-grade TCC and squamous (3 cases), glandular (3 cases), small-cell (one case), and sarcomatoid (one case) differentiation foci. The histological grade (WHO 1973) was G1 in 8 cases (9.8%), G2 in 57 (69.5%), and G3 in 17 (20.7%); and (USIP/WHO 2004) low grade in 43 (52.4%) cases, and high grade in 39 (47.6%). Regarding stage, 3 (3.6%) showed a pTa stage, 42 (51.2%) pt1, 9 (11%) pt2, 24 (29.3%) pt3, and 4 (4.9%) pt4. Nodes involvement was confirmed in 4 of the 18 cases where lymphadenectomy was performed. Invasion of the renal parenchyma was observed in 22 (26.8%) cases, vasculolymphatic invasion in 16 (19.5%), and tumor necrosis in 16 (19.5%).

In 30 cases (36.6%), p53 overexpression was observed, MIB-1 overexpression in 33 (40.2%), and PCAM-1 overexpression in 16 (19.5%). The percentage mean of immunostaining
for p53 was 15.3 ± 25.5 (0–100) and 8.8 ± 11.8 (0–67) for ki-67. Since the ki-67 index was defined in percentage terms for each case, its qualitative distribution by sections was: 49 (59.8%) cases between 0 and 5, 17 (20.7%) between 6 and 12, 10 (12.2%) between 13 and 25, and 6 (7.3%) ≥ 25. The median of this index was 4 (IQR: 6.2–11.4). MIB-1 overexpression was defined for the survival analysis as positive staining in ≥6% of the evaluated nuclei. Table 1 shows the association between molecular variables, grade, and stage. The mean follow-up was 46.8 ± 38.5 (4–172) months. During that time, we registered the death due to the disease of 30.5% of the patients who started the study, the median survival reaching 57 months, with a 95% CI for this estimation of 44–63 months (Fig. 1). The univariate analysis revealed important observations (Table 1). Those patients with smaller tumors (≤ 30 mm maximum diameter) have a lower risk of death due to cancer (HR: 0.374 [0.149–0.938]; p = 0.028). No difference in survival was observed among patients with exclusively pyelic tumors vs. those with uretero-pyelic tumors (HR: 0.903 [0.398–2.047]; p = 0.8).

The presence of a mixed histology entailed a greater cancer-specific mortality risk despite the small number of lesions (HR 5.53 [2.118–14.44]; p < 0.0001). Flat-growth tumors also had a poorer prognosis than those with a papillary growth pattern (HR 0.16 [0.071–0.361]; p < 0.0001). Patients with high-grade tumors had worse survival rates than those with low-grade tumors (HR: 8.163 [2.79–23.88]; p < 0.0001). Similarly, those with infiltration of the muscular layer of the renal pelvis wall in the nephroureterectomy specimen, or even in a deeper layer (pT2-pT4), showed worse cancer-specific survival than those with no infiltration of the muscular layer (pTa-pT1) (HR 2.77 [1.22–6.287]; p < 0.0106). Tumors infiltrating the renal parenchyma also showed worse survival, though this did not reach statistical significance (HR: 0.491 [0.22–1.094]; p = 0.074). The detection of lymphovascular invasion in the surgical specimen also determined a poorer prognosis (HR: 0.394 [0.169–0.92]; p < 0.0001). Finally, the presence of tumor necrosis also conferred worse survival rates (HR: 5.53 [2.118–14.44]; p < 0.0001). With regard to the molecular markers tested (Fig. 1, Table 1), p53 overexpression implied worse cancer-specific survival in the univariate analysis (HR: 0.264 [0.116–0.6]; p = 0.0006), as well as MIB-1 overexpression (HR: 0.319 [0.14–0.725]; p = 0.0038) or PECAM-1 overexpression (HR: 0.318 [0.14–0.721]; p = 0.0036).
The multivariate analysis using Cox’s regression model with the stepwise method showed that independent prognostic variables in this study had a high histological grade (USIP/WHO 2004) (HR: 4.204 [1.282–13.791], p = 0.0718), p53 overexpression (HR: 2.803 [1.221–6.437], p = 0.0151) and a flat-growth pattern (HR: 2.515 [1.049–6.029]; p = 0.0388). Besides, the presence of histology with differentiation foci different from conventional TCC (HR: 2.727 [0.997–7.456]; p = 0.0507) is at the very limit of statistical significance to be also considered as an independent variable for prognostic prediction (Table 3).

Discussion

TCC of the renal pelvis is an aggressive malignancy, largely because of the difficulties in establishing an early diagnosis. This poor survival is particularly unfavorable to patients with high-grade pT3-pT4 tumors which have an elevated risk of showing vascular invasion, even without metastasis or lymphatic node invasion.27-29 Many studies have been undertaken to identify preoperative (clinical), intraoperative (surgical), or postoperative (histological and molecular) prognostic factors. For decades, we have known that predictive clinical factors do not accurately stratify the patients’ risk before the definitive treatment. Furthermore, the relative rarity of this disease makes prospective studies scarce, most of the existing ones being retrospective either by a single institution or multicenter ones.30

The histopathological study of the nephroureterectomy specimen is the cornerstone of prognosis prediction in these patients, so variables such as tumor extension (pT and pN categories), histological grade, and the architectural pattern are the main parameters on which the decision for adjuvant chemotherapy and follow-up for the early detection of recurrence or progression are based.31 At least 4 multi-institutional studies have confirmed the importance of growth patterns as an independent prognostic factor,4,31-33 sessile growth being associated with worse survival. This finding is also reproduced in our experience. Most studies do not cover histologies different from pure TCC, but it has been suggested that these non-conventional histological varieties do not affect the response to adjuvant systemic chemotherapy.34,35 Our study did not use systemic chemotherapy and identified mixed histology as an unfavorable independent predictive variable, an observation concurrent with the identification of different molecular subtypes.36 Finally, histological grade is possibly the only main prognostic factor which can be reliably assessed both pre- and postoperatively.37,38 The binary classification into low and high grade has been validated as a simple predictor and with a broad agreement among pathologists for TCC-UUT.32,37,38

Another morphological criterion frequently identified in studies as an independent predictor is lymphovascular invasion, both in patients with negative nodes45-47 and with node involvement.46 In our experience, this factor was identified in the univariate study, but it did not persist in the multivariate one. The same applied to the size of lesions45 or with the presence of tumor necrosis, sometimes identified as independent,43,44 but not always.45 Some studies have shown that ureteric location can involve a
poorer prognosis than pyelic location.\textsuperscript{29,46} It has been suggested that the renal parenchyma has a protective effect and that the absence of this structure at the urethral level favors local involvement.\textsuperscript{29} In our experience, none of these 2 observations was reproduced. Ureteral involvement is an unimportant predictor. The infiltration of the renal parenchyma implies a poorer prognosis, but without reaching statistical significance.

Several studies have been recently published by major multicenter European and American groups which define nomograms for predicting the clinical evolution of these patients and which, in addition, have been validated in different scenarios.\textsuperscript{51,47-50} These studies gather a vast amount of information and can help with clinical decision-making. Nomograms are superior in prognostic prediction than any of the clinical variables which they incorporate. Nonetheless, the accuracy of these nomograms does not exceed 80\%,\textsuperscript{31,47,49} possibly due to the limitation derived from the retrospectively collected information. As if this were not enough, none of them includes molecular elements.\textsuperscript{50}

The fact that radical surgery severely affects the renal function implies that only a minority of the operated patients receive adjuvant chemotherapy.\textsuperscript{51,52} A reliable pre-operative biopsy which enabled the identification of a molecular signature associated with poor prognosis would help to develop better therapeutic strategies aimed at

**Table 3** Multivariate analysis of predictors of cancer-specific survival.

<table>
<thead>
<tr>
<th>Variable</th>
<th>(B) coefficient</th>
<th>ES</th>
<th>Chi-square</th>
<th>(P) value</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>High histological grade (USIP)</td>
<td>1.4361</td>
<td>0.6061</td>
<td>5.6142</td>
<td>0.0178</td>
<td>4.204</td>
<td>1.282–13.791</td>
</tr>
<tr>
<td>p53 overexpression</td>
<td>1.0307</td>
<td>0.4242</td>
<td>5.9049</td>
<td>0.0151</td>
<td>2.803</td>
<td>1.221–6.437</td>
</tr>
<tr>
<td>Flat-growth pattern</td>
<td>0.9222</td>
<td>0.4462</td>
<td>4.2716</td>
<td>0.0388</td>
<td>2.515</td>
<td>1.049–6.029</td>
</tr>
<tr>
<td>Non-TCC convencional histology</td>
<td>1.0031</td>
<td>0.5132</td>
<td>3.819</td>
<td>0.0507</td>
<td>2.727</td>
<td>0.997–7.456</td>
</tr>
</tbody>
</table>
stratifying risks and at using neoadjuvant chemotherapy. We know that there is a great similarity between elements involved in the cell cycle and proliferation markers in TCC of the UUT and of the urinary bladder. P53 overexpression is associated with advanced tumor stage and poor prognosis, but this conclusion is not shared by all studies. A recent meta-analysis on p53 accumulation and TCC-UUT supported the significant value of this marker for prognostic definition. Nevertheless, this study had a certain weakness since its conclusions were based on a univariate analysis of retrospective studies. Our experience strongly supports these findings and shows the independent prognostic value of p53, which is not associated with histological grade or tumor stage.

There is also great controversy with regard to MIB-1 overexpression. Many studies have shown that the proliferation index is a major predictor in TCC-UUT. However, not all studies have confirmed this finding. Possibly, those studies including a high proportion of low-grade tumors have less power; a fact which also occurred within this series. Besides, there is little uniformity in the cut-off point defining overexpression, which ranges from 10 to 30%. On the other hand, the presence of neofomed vessels is considered an element favoring tumor growth, immunostaining with CD31 being established as an evaluator of angiogenesis in this type of tumors. In our experience, MIB-1 and PECAM-1 overexpression implies a poorer prognosis in renal pelvic carcinoma, but they are not independent predictors.

The fact that evaluation is centralized to one single pathologist, the prospective nature of the study, and the application of a homogeneous treatment based exclusively on radical surgery are valuable elements. The main limitation resides in the magnitude of the series which determines the number of independent variables.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**References**

Molecular markers in carcinoma of the renal pelvis


