

# MIB-1 and p53 as markers of aggressiveness in superficial transitional cell carcinoma of the bladder

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## Abstract

**Aim:** To assess the prognostic value of the MIB-1 and p53 immunostaining in relation to tumor recurrence and progression in the superficial (pTa-pT1) transitional cell carcinoma (TCC) of the bladder.

**Material and methods:** This is a retrospective study of 98 cases of patients with superficial papillary transitional cell carcinoma of the bladder, diagnosed in the Coimbra Regional Oncology Center (I.P.O.F.G.) between 1992 and 1996, by analysis of the expression of the cellular proliferation marker (MIB-1) and p53 in neoplastic cell populations, using immunohistochemical methods in paraffin embedded tissues. The results were analyzed in relation to recurrence and progression of the disease and in relation to conventional prognosis factors such as gender, age, histological grade and pathological stage. Patients were stratified for both markers into two groups for time-event analysis, according to the median value of nuclei stained. Patients with nuclear expression below the median value of the score were considered negative in the statistical analysis. In order to determine the prognostic significance of nuclear immunoreexpression of MIB-1 and p53, its relationship with recurrence and progression was studied, using univariate and multivariate analysis (Cox's proportional hazards model).

**Results:** There was a significant association between positive MIB-1 and p53 and histological grade ( $p=0.0057$  and  $p<0.0001$ , respectively) and with stage ( $p=0.013$  and  $p=0.027$ , respectively). The median nuclear expression of p53 was 6%, and that of MIB-1 was 7%. There was a significant association between proliferation and p53 nuclear overexpression ( $p<0.001$ ). The probability of remaining free from recurrence and of progression of the disease ('Log Rank Test') was significantly lower in patients with MIB-1 and p53 scores above 6% and 7%, respectively. The univariate analysis showed that positive values for MIB-1 ( $p=0.001$ ), p53 ( $p=0.0141$ ) and stage ( $p=0.0369$ ) were significantly associated with the risk of tumor recurrence. The

multivariate analysis indicated that immunostaining of MIB-1 ( $p=0.058$ ) was the only factor with independent prognostic value in relation to the recurrence of the disease.

**Conclusion:** The immunohistochemical evaluation of MIB-1 has independent prognostic value in cases of superficial TCC of the bladder, predicting a significant increase in the risk of recurrence.

**Key words:** superficial bladder carcinoma, p53, MIB-1, prognostic factor, immunohistochemistry

**Abbreviations:** TCC, transitional cell carcinoma; pT, pathological stage; TUR, transurethral resection; BCG, Bacillus Calmette-Guérin; LI, labeling index.

## Introduction

Transitional cell carcinoma (TCC) of the bladder represents 90% of all primary bladder tumors, and is mostly found in patients over 50 years of age, more commonly male (1,2,3). Around 80% of them are diagnosed in the superficial (non-invasive) phase (4), however, 80% of superficial papillary TCCs recur within a period of six to twelve months, and 20% of these progress in stage or grade (5,6). Therefore, there is a need to establish prognostic factors that could help to identify tumors likely to develop more aggressive behavior and which require different clinical follow-up.

Several parameters have been used to predict the biological behavior of these tumors, including pathological stage (pT), histological grade, size of tumor, and multifocality (7,8). However, TCCs have a somewhat unpredictable biological behavior, and the systems of tumor grading and staging are unable to adequately predict the development of the disease (9).

The cellular mechanisms underlying the heterogeneous behavior of TCCs are mostly unknown. Recent advances in tumor biology have suggested the possible existence of markers that could form the basis for a more accurate classification of tumors (10), in order to establish the biological behavior of the initial lesion and determine if the patient is at risk of recurrence or of recurrence with progression.

In order to try to identify patients with superficial TCC of the bladder that recur and progress (in grade and in stage), we analyzed the expression of the cell proliferation marker (MIB-1) and of p53 in neoplastic cell populations, using immunohistochemical tech-

nique in paraffin embedded sections (1,5,11). The reason for the study of these two markers is due to the fact that, firstly, p53 is a 'major' regulator of the cell cycle, and, secondly, p53 gene product alteration and cell proliferation may be assessed in the same material using the same methodology (12).

MIB-1 identifies the protein complex 345-395 KD, which is expressed in all phases of the non-G0 cycle and is thus a marker of cells that participate in cell proliferation (13).

P53 is a tumor suppressor gene located in the short arm of chromosome 17, and codifies a nuclear phosphoprotein that acts in cell proliferation regulating the transcription of DNA. By stopping the cycle in G1, the p53 protein permits the repair mechanisms to act upon spontaneous or induced errors in DNA. If these mechanisms fail, p53 may set into motion apoptotic events that lead to the destruction of the damaged cell (14).

## Material and Methods

### Cases

We studied 98 cases of patients with papillary superficial TCC of the bladder diagnosed in the Coimbra Oncological Center (IPOFG) between 1992 and 1996. The 73 men and 25 women were aged between 22 and 98 years (mean age 68.8).

Tumor staging was made according the TNM System(15) and was based upon a combination of clinical and pathological data, namely cystoscopy, ultrasound, urography, TAC, cytology and the histological study of biopsy material obtained by transurethral resection (TUR) to determine the absence (pTa) or presence (pT1) of invasion of the 'lamina

propria' or the detrusor muscle (pT2 to pT4). The absence of histological representativity of the muscle layer implies the tumor non-staging, and this kind of tumor are thus considered to be non-assessable (NA), although it is classified as superficial on the basis of clinical-pathological data (16). A normal urothelium was observed in 50 cases.

The histological grade was determined using WHO recommendations (2). The distribution of cases by stage and grade is presented in Table 1.

Initial therapy consisted of TUR in 98 cases. Of these, 59 were subjected to intravesical BCG, 3 to intravesical chemotherapy, 1 to radiotherapy and the remaining 35 to clinical surveillance alone.

The mean follow-up was 30.4 months (2 to 67 months) and consisted of periodical and routine cystoscopies and cytology in accordance with defined procedures. 30 cases (30%) recurred, of which 9 (30%) progressed, 3 in grade (from grade 1 to grade 2) and 6 in stage (2 from pTa to pT1; 4 from pT1 to pT2). The mean time of recurrence and progression of the disease was 13.6 months (3-29 months) and 15.6 months (5-28 months) respectively.

Recurrence and progression were defined in accordance with the literature: recurrence as the development of a new tumor growth, and progression as a new tumor growth with a higher histological grade and/or pathological stage than the original one, both after three months of the initial TUR.

## Histology

TUR material was fixed in 10% formol, embedded in paraffin and stained with haematoxylin-eosine, and was studied by two independent observers that determined the pathological grade and stage.

## Immunohistochemistry

The immunohistochemical method employed was the Streptavidine-Biotine complex (STREPTABC/HRP), with use of the automatic Techmate 500 apparatus. The material was incubated for one hour with the antibodies Ki-67 and p53 (DAKO A/S; Denmark) in a dilution of 1/65 and 1/1000 respectively. Antigenic retrieval was carried out in microwaves 2 x 5 mn. As positive controls we used sections from a human tonsil and squamous cell carcinoma for Ki-67 and p53, respectively. As internal negative control, non-

GRADE	STAGE			Total
	pTa	pT1	NA	
I	11	4	20	35
II	9	23	21	53
III	0	6	4	10
<b>Total</b>	20	33	45	98

**Table 1:** Distribution of patients by stage (UICC-TNM) and by histological grade (WHO classification)

epithelial cells were used (lymphocytes, stromal cells and endothelial cells).

## Assessment of the MIB-1 and p53 immunostaining

In the quantitative analysis of the immunostaining, we selected the areas with higher positivity, on regardless if it corresponds to non-invasive or invasive areas of the tumor. In each case, more than 300 cells were counted in the selected areas. Nuclei of a dark brown color or diffuse and grainy shape, were considered positive.

MIB-1 and p53 labeling indexes ( LI ) were estimated as the number of positive nuclei divided by the total number of nuclei scored, and, expressed as a percentage.

The cut-off value of the immunostaining for statistical analysis was determined by the median distribution of each variable. Patients with LI below the median value were considered MIB-1 and p53 negative, and with LI equal or above the median value were considered MIB-1 and p53 positive.

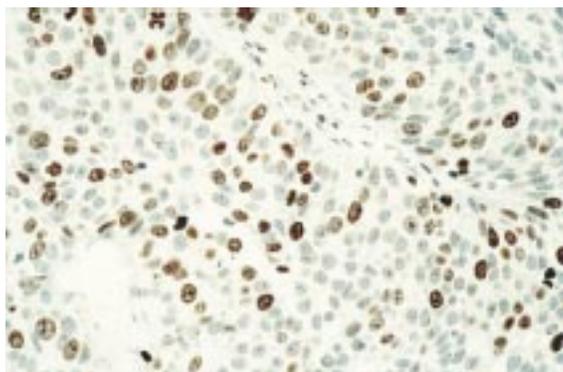
## Statistical Analysis

MIB-1 and p53 nuclear immunostaining and clinical-pathologic data were analysed in cases with and without recurrence of disease, and with and without progression (in grade and in stage).

Continuous variables were compared using variance analysis and the frequency distributions were compared by the Qui-squared test.

The correlation between the MIB-1 and p53 LI was estimated.

The Kaplan-Meier graphs and the logrank test were used to assess the association of the expression



**Figure 1.** Nuclear immunostaining: MIB-1 positive x 400

of MIB-1 and p53 in accordance with the recurrence and progression free intervals. Recurrence and progression free intervals of the disease were defined: as the time between the TUR date and the recurrence or the last clinical examination; and as the time between the TUR date and the progression or last clinical observation, respectively.

The prognostic value for the recurrence and progression of the disease was determined by the univariate analyses for each of the factors individually: gender, age, histological grade, pathological stage, MIB-1, and p53 immunostaining. The factors that had prognostic value in the univariate analysis were tested by multivariate analysis. The independent prognostic value of each factor for risk of recurrence was determined by the Cox proportional hazards model. The odd ratios (ORs) and confidence intervals (CIs) were estimated.

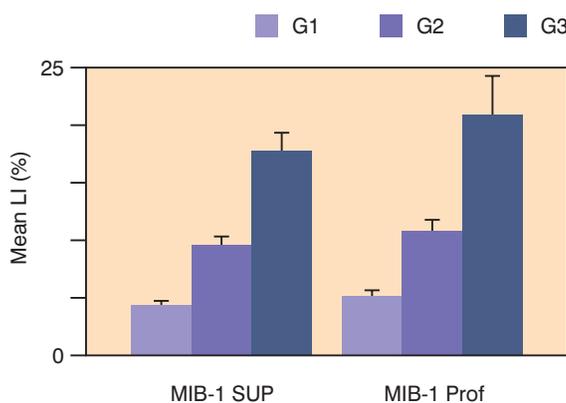
A  $p < 0,05$  was considered as indicative of statistical significance.

## Results

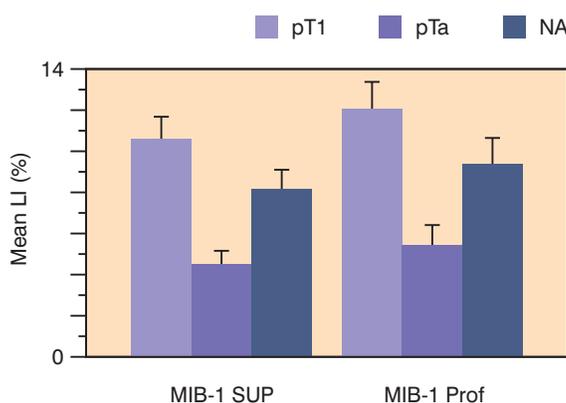
### MIB-1 expression and association with Stage and Grade

In papillary tumors Ta-T1, nuclear staining was found in the basal layers of some tumors, while in others, it was scattered throughout the whole thickness (figure 1).

Tumor MIB-1 labeling index (LI) had values varying between 0.6% and 26.3% (mean LI=8.7%; median=7%; standard error=0.684). The labeling index assessed on the superficialness of the tumor had values which varied between 0.6% and 24% (mean LI=7.8%, standard error=0.6) and in the deep tumor, the values varied between 0.6% and 34% (mean LI=10.0%; standard error=0.8%).



**Figure 2 -** Mean labeling index (LI) for MIB-1 as a function of the grade (who). MIB-1 Sup: labeling of superficial layers of tumor; MIB-1 Prof: labeling of deep levels of tumor



**Figure 3 -** Mean labeling index (LI) for MIB-1 as a function of the stage (TNM). MIB-1 Sup: labeling of superficial layers of tumor; MIB-1 Prof: labeling of deep levels of tumor

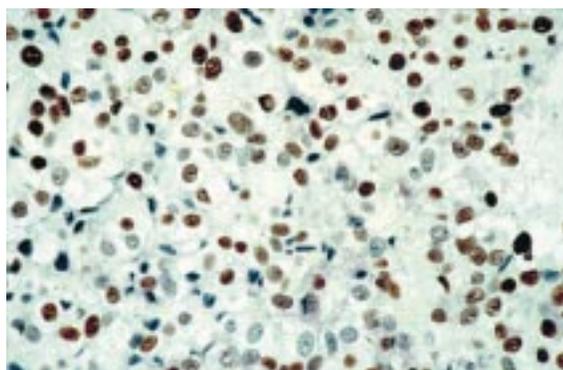
The labeling index in the normal urothelium had values that varied between 0,0% and 7,0% (mean LI=0.7%), with labeling observed in the proliferative zone next to the basal layer.

We observed a significant association between the histological grade ( $p=0.0057$ ) and stage ( $p=0.013$ ), and the percentage of stained cells (figures 2 and 3), even when different proliferation indices were observed for each stage and each grade.

The median value (7%) was used as a cut-off for other statistical analysis.

### P53 expression and association with Stage and Grade

Tumor p53 labeling index had values that varied between 0% and 80% (mean LI, 11.84; median, 6%;



**Figure 4** - Nuclear immunostaining: p53 positive x 400

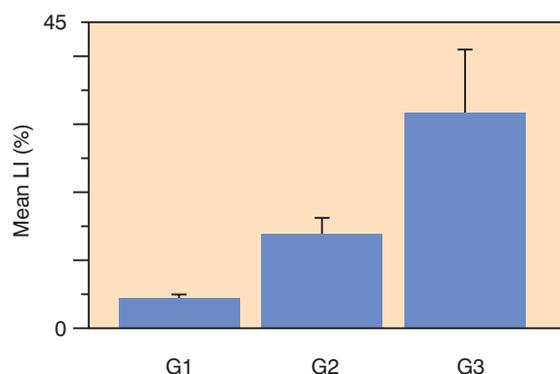
standard error, 1.1762). Of the 50 cases in which a normal urothelium was observed, 18 had p53 overexpression, with LI varying between 0.3% and 6.3% (mean LI, 0.532). Figure 4 illustrates p53 nuclear expression.

A significant association was observed between histological grade ( $p < 0.0001$ ) and the percentage of stained cells, which was also observed with stage (stages pTa and pT1 by the Anova Postoc test, with the value of  $p = 0.027$ ), although each stage and histological grade reveal a variety of staining intensities (figures 5 and 6). The median value (6%) was used as a cut-off for other statistical analysis.

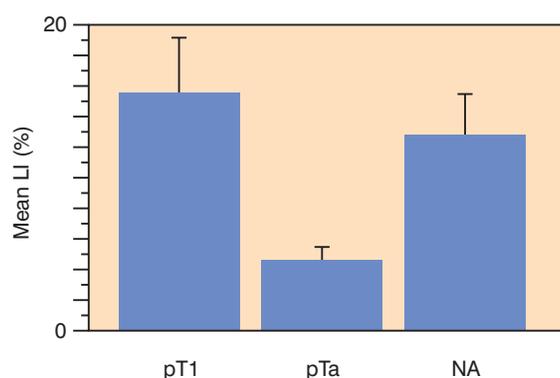
A correlation was observed between p53 overexpression and MIB-1 (correlation coefficient = 0.499;  $p < 0.001$ ).

### MIB-1 and p53 expression and recurrence and progression free intervals of the disease

Recurrence free and progression free curves were drawn using the Kaplan-Meier method, with a cut-off value of 7% for MIB-1 and of 6% for p53. This allowed patients to be categorized into two groups with different staining patterns for MIB-1 and p53, so that recurrence and progression free intervals could be compared. The probability of recurrence and progression was shown to be greater in patients with positive MIB-1 and p53 than in those with negative, and the difference was statistically significant ('log rank test'). Table 2 indicates the p value for each category of the Log Rank Test. Figures 7, 8, 9 and 10 show the curves of recurrence and progression free interval for MIB-1 and for p53, respectively.



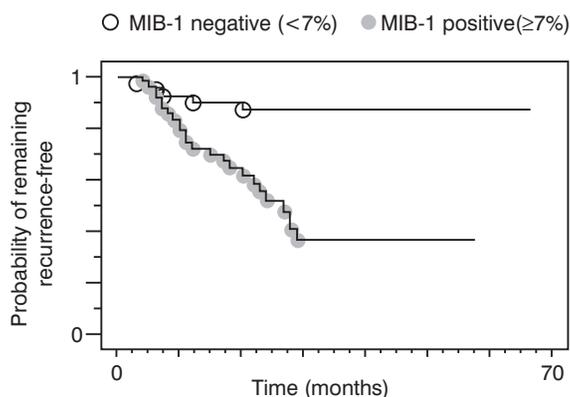
**Figure 5** - Mean labeling index (LI) for p53 as a function of the grade (WHO)



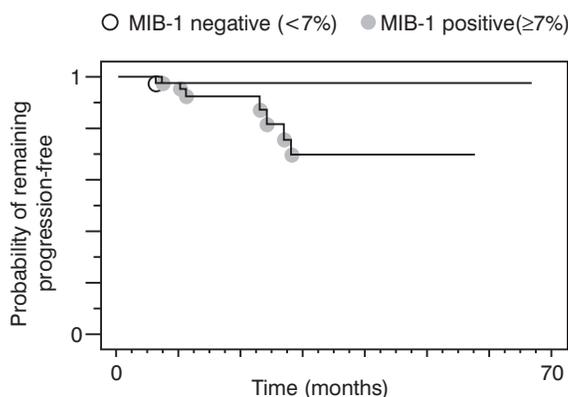
**Figure 6** - Mean labeling index (LI) for p53 as a function of the stage (TNM)

	TCC	Recurrence	Progression
	(n = 98)	(n = 30)	(n = 9)
<b>P53</b>			
< 6%	54	9	2
≥ 6%	44	21	7
Recurrence P value	0.0003		
Progression P value	0.0164		
<b>MIB-1</b>	(n = 98)	(n = 30)	(n = 9)
< 7%	45	5	1
≥ 7%	53	25	8
Recurrence P value	< 0.001		
Progression P value	0.0100		

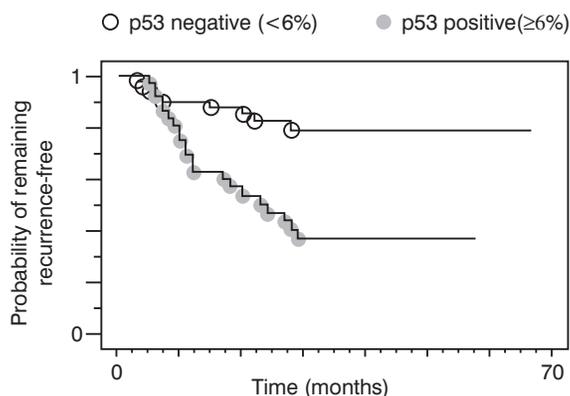
**Table 2:** The prognostic value of p53 and MIB-1 cutoffs for recurrence and progression of the disease, according to Kaplan-Meier estimates and Log Rank Test comparison



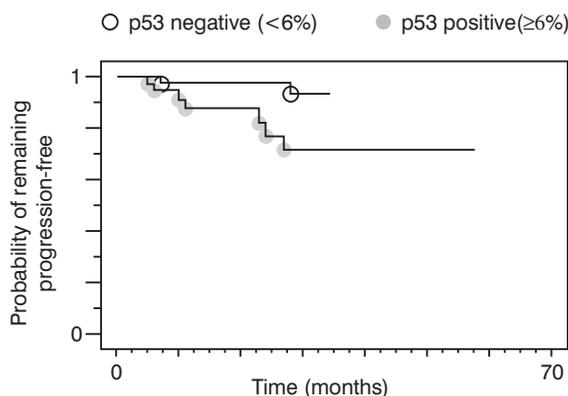
**Figure 7:** Kaplan-Meier Curves illustrating the disease recurrence-free intervals for MIB-1 positive and negative tumors. Log Rank Analysis comparing these two groups shows a significant difference in the probability of recurrence ( $p < 0.001$ )



**Figure 9:** Kaplan-Meier Curves illustrating the disease progression-free intervals for MIB-1 tumors positive and negative tumors. Log Rank analysis comparing these two groups shows a significant difference in the probability of progression ( $p < 0.001$ )



**Figure 8:** Kaplan-Meier Curves illustrating the disease recurrence-free intervals for tumors with and without p53 overexpression. Log Rank analysis comparing these two groups shows a significant difference in the probability of recurrence ( $p = 0.0003$ )



**Figure 10:** Kaplan-Meier Curves illustrating the disease progression-free intervals for tumors with and without p53 overexpression. Log Rank analysis comparing these two groups shows a significant difference in the probability of progression ( $p = 0.0164$ )

## Univariate Analysis

The following variables were significantly associated with recurrence of the tumor: stage ( $p = 0.0369$ ), MIB-1 immunostaining ( $p = 0.001$ ) and p53 immunostaining ( $p = 0.0141$ ). The grade also showed an association with recurrence ( $p = 0.053$ ). The other variables namely, gender ( $p = 0.263$ ) and age ( $p = 0.100$ ), did not reveal any significant association with recurrence.

In the univariate analysis, no variable was significantly associated with tumor progression.

## Multivariate Analysis

The factors which had prognostic value in relation to the recurrence of disease in the univariate analysis were tested by multivariate analysis (Cox proportional hazards model) with the aim of assessing its independent prognostic value. The results are shown in Table 3.

Patients with positive MIB-1 (LI 37%) presented a risk of recurrence of the disease 4.5 times higher in relation to those with negativity for this marker. This is therefore the only factor with independent prog-

nostic value relative to recurrence of the disease ( $p=0.0058$ ). With regards to p53 overexpression, a small increase in risk was observed (OR=2.039) though not significant ( $p=0.140$ ).

## Discussion

In this study, we assess the prognostic value of MIB-1 and p53 expression, and of clinical-pathologic parameters (grade and stage) in relation to the recurrence and progression of the tumor in a group of 98 patients with superficial TCC of the bladder (pTa–pT1), in a mean follow-up period of 30.4 months.

Our results show that the grade and stage of the tumor has prognostic value in relation to the recurrence of superficial TCCs, which is in agreement with the literature (17,18,19,20,21,9,22). However, the biological behavior of the TCCs of the bladder is generally rather unpredictable, which would suggest that groups defined by morphologic methods of grading tumor malignancy are heterogeneous with regards to biologic behavior (9). The initial therapy for superficial TCCs is usually selected on the basis of clinical-pathologic parameters, which has two important limitations: intra- and inter-observer variation in the assessment of the grade and stage; the use of additional therapy, especially the BCG, which conditions the predictive capacity of the response to therapy by modifying the natural history of the TCCs, thus making other biological parameters necessary (23,24).

We demonstrated using univariate analysis that the MIB-1 expression and p53 overexpression were significantly associated with the recurrence of disease. However, according to the multivariate analysis, MIB-1 was the only factor which has independent prognostic value. Tumors with MIB-1 LI higher than 7% had a risk approximately 4.5 times greater of recurrence in relation to those with LI lower than 7%. As for progression, we did not observe any significant association with MIB-1 and p53 expression in the univariate analysis. However, when we categorized the labeling index using the median as a cut-off (MIB-1 median=7%; p53 median=6%), and drew curves showing the progression-free intervals using the Kaplan-Meier method, we observed that positivity for these markers was significantly associated (Log Rank Test) with progression, and that, of the 9 tumors that progressed, 8 were MIB-1 positive, and 7 were p53 positive. These findings suggest that the use of a cut-

Factors Investigated	P Value	OR	95% Confidence Interval
MIB-1	0.0058	4.574	1.553– 13.469
p53	0.1400	2.039	0.7916– 5.253

**Table 3:** Contribution of certain potential prognostic variables with regards to recurrence by Cox's Proportional Hazards Regression Analysis Model.

off point for these markers is a method for predicting the progression of superficial TCCs.

Our results are in agreement with those described in various studies, and suggest that proliferation activity (MIB-1) and p53 overexpression are independent prognostic factors in bladder TCC. However, it must be stressed that the clinical series were heterogeneous, and included patients with both superficial and invasive tumors, and that the multivariate analysis that includes the interactive effects of these two markers was not carried out in a significant number of studies.

We also observed that MIB-1 and p53 have a significant association with pathological stage and with histological grade, which is in accordance with the literature: it occurs in superficial tumors (25,26,27,28) as well as in invasive tumors (12,22,29), for both markers.

In accordance with the literature, p53 expression detected by immunohistochemistry may be due to different situations, and is usually associated with p53 gene mutations (30,31). However other mechanisms can be associated with overexpression as non-mutational stabilization of p53 protein brought about by cell oncogene products (such as MDM2, which is generally amplified in sarcomas) and some viral proteins that connect and inactivate the wild p53, prolonging its half-life with consequent nuclear accumulation (32); there may also be an increase in the wild protein p53 as a normal response to DNA lesion (33). In contrast, 15-20% of tumors with p53 gene mutation do not have overexpression in the immunohistochemical analysis (34). Deletions as well as 'non sense' mutations may prevent the formation of p53, and thus, the loss of activity of the p53 protein is not accompanied by nuclear accumulation (34,35).

Although we have demonstrated that the presence of alterations of the p53 protein detected by immunohistochemical methods is clinically relevant, the lack of agreement between immunohistochemi-

cal methods and the analysis of p53 mutations indicates that the comparative analysis of the mutation and p53 overexpression in the tumor progression is necessary to assess if the mutation is the cause of the overexpression detected.

p53 gene mutation may be caused by carcinogenic agents such as one of the components of tobacco smoke. It has been demonstrated that smoking increases the frequency of p53 mutation in patients with bladder cancer and may therefore contribute to the prognosis (5,36). p53 overexpression in this study is not however related to the history of smoking habits, due to the absence of data.

The criteria for recurrence and progression vary from author to author and is dependent of the selection of the patient population, that is, patients with superficial disease versus those with invasive disease. The criteria for recurrence and progression that we used was the following: recurrence as the development of a new tumor growth, and progression as a new tumor growth with a higher histological grade and/or pathological stage than the original one, both after three months of the initial TUR. The criteria adopted was used by some authors irrespective of whether the tumors were superficial or invasive (32, 37, 35).

In this study, the cut-off value clinically useful in a population of patients with superficial TCC was 7% for MIB-1 and 6% for p53. The cut-off value mentioned in the literature varies, for MIB-1 between 5.35% (28) in superficial tumors and 20% in invasive tumors(12); for p53, some studies consider the value of 10% (34, 38, 25, 32) and others 20% (5,27) in superficial pT1 tumors; in other studies of superficial and invasive TCCs, the values of 10% (29) and 20% (12) are considered equally, and a value of p53 < 0% has been considered as positive in pTa grade 1 tumors (35).

The use of the cut-off value in the categorization of the variables is of importance in clinical practice and in the comparison of data reported in the literature. The determination of the cut-off varies between authors. In some studies, it is defined for p53 as the level of immunostaining associated with gene mutation. As there is no precise correlation between the p53 gene mutation and p53 immunostaining, we, like other authors, used another method to define the cut-off value; we studied the distribution of the variables in our population and categorized their labeling index using the median value as cut-off (12,28).

Although the mean follow-up period of our study was relatively short, in 30.6 months, 33% of tumors recurred, and of these, 30% progressed, which is in accordance with the literature which states that 25% of tumors progress clinically during the first three years after presentation and only 7% in the following seven years(39). Other studies confirm that superficial TCCs rarely progress in long follow-up periods (5,6,40, 41,42) which suggests that valid conclusions may be drawn about the prognostic significance of various markers of urethelial tumors after a relatively short follow-up period (43).

Further studies with a larger number of patients with superficial TCC are needed to assess p53 overexpression and MIB-1 expression in tumor cells detected by immunohistochemical methods, as independent indicators of prognostic progression. If the prognostic value of these markers (which can be easily assessed on routine preparations with limited equipment) is confirmed, strategies for differentiated clinical follow-up should then be discussed.

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