

## Altered p53 and pRb Expression Is Predictive of Response to BCG Treatment in T1G3 Bladder Cancer

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**Abstract.** *Background: The aim of this study was to determine the prognostic value of p53 and retinoblastoma protein (pRb) expression in patients with stage T1 grade 3 (T1G3) bladder cancer (BC) treated by transurethral resection of bladder tumour (TURBT) and intravesical instillations of bacillus Calmette-Guerin (BCG). Materials and Methods: p53 and pRb expression were independently recorded within a homogeneous series of 27 patients. Fisher exact test and the log-rank test were carried out, along with Kaplan-Meier survival analysis. Results: Sixteen tumours showed altered p53 expression, while 14 showed altered pRb expression. Overall, 7 tumours showed normal expression of both markers, 10 altered expression of one of the two markers, and 10 altered expression of both markers. Only altered pRb expression was an independent predictor of both recurrence ( $p=0.037$ ) and progression ( $p=0.018$ ); altered expression of both markers was a strong predictor ( $p=0.001$ ) of progression. Conclusion: This is the first study demonstrating that altered p53 and pRb expression are predictive of T1G3 BC response to BCG treatment. These findings provide grounds for inclusion and prospective validation of these markers in the decision-making process for treating BC.*

Although transurethral resection of bladder tumour (TURBT) followed by intravesical instillation of bacillus Calmette-Guerin (BCG) is considered the first-line treatment for stage T1 grade 3 (T1G3) bladder cancer (BC) in both European and American urological guidelines (1, 2), its long-term

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outcome is unpredictable: one third of patients never recur or progress, one third requires deferred cystectomy and another third eventually dies of this disease (3). Prediction of response to BCG treatment is therefore a major clinical issue.

Since traditional pathological features such as tumour size and number, concomitant carcinoma *in situ* and depth of lamina propria invasion have been shown to be of little value in routine clinical practice (4), the search for prognostic factors has moved to molecular markers of cell cycle regulation and cell proliferation, such as p53, p21, and Ki-67. While p21 and Ki-67 failed to predict response to BCG treatment in multivariate analysis (5), the prognostic value of p53 remains controversial (4).

Following the observation that mutations of *p53* and retinoblastoma (*RB*) tumour-suppressor genes may have cooperative carcinogenic effects in mice (6), attempts were made to determine whether p53 and RB synergistically promoted BC progression in humans. The few studies focusing on this issue yielded controversial results, probably due to inclusion of tumours of different grades and stages (often  $\geq T2$ ), and even having received different primary or adjuvant treatments (7, 9-14). Even less information is available on the utility of p53 and pRB expression in predicting response to BCG treatment, as we found only one study testing such issue in a heterogeneous population of superficial bladder tumours (15), and none testing it in the subset of patients with T1G3 BC. The present study therefore aimed to determine whether altered p53 and pRb expression was predictive of response to BCG treatment in the sub-population of T1G3 tumours, those electively treated with BCG and yet at higher risk of progression.

### Materials and Methods

*Patients, study design and specimen characteristics.* The study population consisted of 27 patients (25 men and 2 women) who underwent complete TURBT. Bladder samples were formalin fixed,

paraffin embedded, and diagnosed as T1G3 BC on light microscopy examination of haematoxylin-eosin slides. All cases were independently reviewed by the two study reference pathologists, who were unaware of clinical data and the original diagnosis. Patients with TaG3 tumours or with concomitant TisG3 tumours were excluded. To avoid the risk of understaging, cases where the bladder muscle was not clearly identifiable were excluded as well. After TURBT, all patients received intravesical instillations of BCG (Pasteur strain, 75 mg in 50 ml saline) once a week for 6 consecutive weeks, had negative urine cytology and negative (*i.e.* absence of tumour) restaging TURBT (including random bladder biopsies) 6 weeks after having completed the BCG induction cycle, and were then scheduled for BCG maintenance (one instillation every 3 months for 1 year).

Follow-up consisted of urine cytology and cystoscopy every 3 months for the first two years, every 6 months for the third year, and then yearly. Intravenous pyelography (IVP) was performed every second year to rule out upper tract disease. Tumour recurrence was defined as pathological evidence of disease at bladder biopsy or TURBT, whereas progression was defined as pathological shift to muscle invasive disease at bladder biopsy or TURBT or the appearance of metastasis.

**Immunohistochemistry.** Serial sections 4 µm-thick were cut, deparaffinized in xylene, rehydrated in graded ethanol solutions and washed for 5 minutes with distilled water.

p53 and pRb expressions were assessed by standard biotin-avidin-complex immunohistochemistry using the monoclonal antibodies p53-DO7 (DAKO, Glostrup, Denmark; dilution 1:50) and Anti-Human Retinoblastoma Gene Product (pRb) clone Rb1 (DAKO; dilution 1:50), respectively. Appropriate positive (BC with known reactivity for the p53 marker, and colon cancer with known reactivity for the pRb marker) and negative (substituting the primary antibody with only antibody diluent) controls were set for comparison with the specimens.

Immunohistochemical analysis was carried out by two pathologists in an independent and blinded fashion. For each specimen, the whole section was examined under light microscopy (×400 magnification) and the number of positive nuclei manually counted in 5 areas and then assessed as a percentage (labelling index). p53 overexpression (positivity) was defined according to the Memorial Sloan-Kettering Cancer Center as a labelling index of at least 20% (8); pRb expression was considered normal when the labelling index was 1% to 50% and altered when the labelling index was 0% or >50% (9).

**Statistical analysis.** Kaplan-Meier curves of disease-free and progression-free survival were tested for significance with the log-rank test, whereas differences in rates were tested with the Fisher exact test. Significance was set at  $p \leq 0.05$ .

## Results

The mean age at diagnosis of the 27 patients included in this study was 69 years (range 57-81 years). The mean follow-up was 60 months (range 15-135).

Ten (37%) patients failed to respond to BCG treatment. In particular, five patients experienced recurrent superficial tumours (2 TaG2, 2 T1G2, and 1 T1G3), not in the areas of

Table I. Prognostic value of clinical variables.

Clinical variable		Recurrence rate	Progression rate
Primary	20 (74%)	7 (35%)	3 (15%)
Secondary	7 (26%)	3 (43%)	2 (29%)
<i>p</i> -Value*		1.00	0.57
Single	12 (44%)	6 (50%)	3 (25%)
Multiple	15 (56%)	4 (27%)	2 (13%)
<i>p</i> -Value*		0.25	0.62
Total size <4cm	12 (44%)	6 (50%)	3 (25%)
Total size >4cm	15 (56%)	4 (27%)	2 (13%)
<i>p</i> -Value*		0.25	0.62

\*Fisher's exact test.

initial T1G3 tumours, 6 to 48 months (mean 18.5 months) after restaging TURBT and were successfully treated by a second BCG treatment (6 weeks' induction cycle, restaging TURBT, 1 year maintenance); one patient found to have a recurrent T1G3 tumour in the area of the initial T1G3 tumour 6 months after restaging TURBT progressed to muscle-invasive disease 12 months after having received a second BCG treatment; four patients experienced direct progression to muscle-invasive disease 6 to 48 months (mean 19 months) after restaging TURBT.

No traditional clinical variable was able to predict recurrence or progression after BCG treatment (Table I).

Sixteen (59%) tumours were p53 positive, while 11 (41%) were negative; 13 (48%) showed normal whereas 14 (52%) showed altered pRb expression (no expression in 6 and overexpression in 8). Overall, 7 (26%) patients presented normal expression of both markers (wild-type), 10 (37%) presented altered expression of one of the two markers (ALT-1), and 10 (37%) altered expression of both markers (ALT-2). Altered p53 expression did not predict recurrence, as the recurrence rate was 38% in p53-positive vs. 36% in p53-negative patients, with the difference in disease-free survival not being statistically significant ( $p=0.92$  according to the log-rank test). Conversely, altered pRb expression predicted recurrence, as the recurrence rate was 57% in patients with altered vs. 15% in patients with normal pRb expression, with the difference in disease-free survival being statistically significant ( $p=0.037$  according to log-rank test). When combining the two markers, the recurrence rate was 29% (2/7) in wild-type patients, 20% (2/10) in ALT-1 patients, and 60% (6/10) in ALT-2 patients; the difference in disease-free survival (Figure 1) did not reach statistical significance ( $p=0.08$  according to log-rank test).

Progression of disease despite BCG treatment occurred in 5 (18.5%) patients and the mean time to progression was 19 months (range 6-48). Altered p53 expression was almost

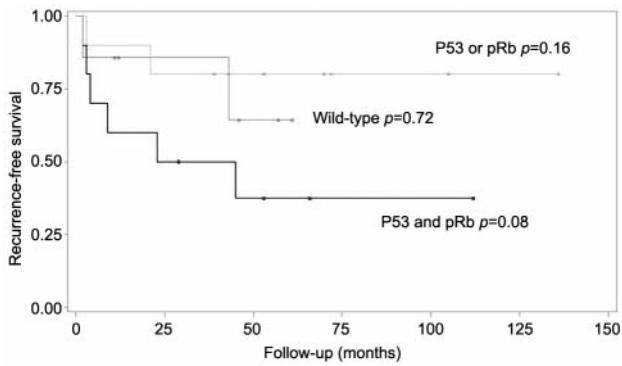


Figure 1. Recurrence-free survival according to p53 and pRb status.

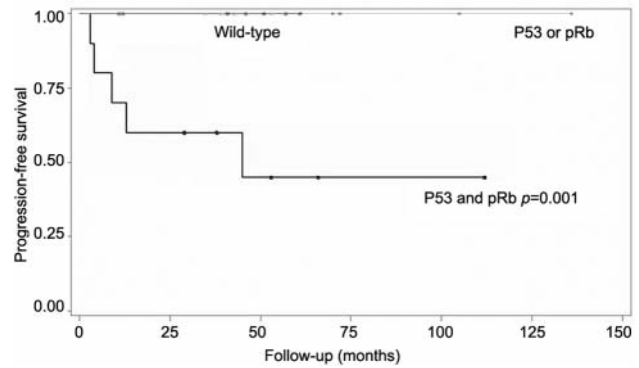


Figure 2. Progression-free survival according to p53 and pRb status.

predictive of progression (31% in p53-positive vs. 0% in p53-negative patients,  $p=0.06$  according to log-rank test), whereas altered pRb expression was clearly predictive of progression (36% in patients with altered expression vs. 0% in those with normal pRb expression,  $p=0.018$  according to log-rank test). When combining the two markers, the progression rate was 0% in wild-type and ALT-1 patients and 50% (5/10) in ALT-2 patients (Figure 2) and this difference was highly significant ( $p=0.001$ ). Such absence of events (progression rate 0%) did not allow for multivariable analysis.

## Discussion

The impact of altered expression of cell cycle regulators on the clinical outcome of muscle-invasive BC has been widely investigated in the last decade. Since altered expression of one or more of these markers has been found to be associated with poorer outcome, the role of p53, pRb, p21 and p27 as prognostic factors in muscle-invasive disease is currently being evaluated in several ongoing clinical trials. Far less work has been carried out with non muscle-invasive BC and much of it has been conflicting, probably because most studies were biased by the inclusion of tumours of different stage (Ta, T1, CIS) and grade (G 1-3), and patients having received different treatment.

To avoid these biases, we tested the prognostic value of p53 and pRb expression in a population of non muscle-invasive BC homogeneous for stage (T1), grade (G3), and treatment (TURBT + intravesical instillation of BCG). Altered p53 expression was not an independent predictor of recurrence; progression occurred in 31% of p53-positive patients vs. none of the p53-negative ones, but this difference did not reach clear statistical significance ( $p=0.06$ ). Conversely, altered pRb expression was found to be an independent predictor of both recurrence ( $p=0.037$ ) and progression ( $p=0.018$ ). Altered expression of both markers was not a clear predictor of recurrence ( $p=0.08$ ), but was a

strong predictor ( $p=0.001$ ) of progression, as 50% of ALT-2 patients progressed as opposed to none of ALT-1 or wild-type patients. Most interestingly, the 31% and 36% progression rates recorded when p53 and pRb were independently evaluated rose to 50% when both markers were altered, but dropped to 0% when only one of them was altered, suggesting that changes involving at least 2 cell cycle regulators are needed to achieve BC progression. These findings are in agreement with those of a recent study (16) testing the prognostic value of altered expression of p53, pRb, p21 and p27 in non muscle-invasive BC and showing that altered expression of at least two of such cell cycle regulators is needed to confer on the tumour a significant increase in progression risk. As a matter of fact, patients with altered expression of only one marker had no greater progression risk than did wild-type patients, whereas patients with altered expression of 2, 3 or 4 markers had significantly higher progression risk ratios (ranging from 9 to 56%). However, this study also presented the potential biases of testing tumours of different stage (Ta, T1, CIS) and grade (G 1-3), and patients having received different treatment.

Little effort has been made to determine the prognostic value of altered p53 and pRb expression in patients treated with BCG. Reviewing the literature, we found only one study (15) addressing this issue and showing that altered p53 and pRb expression did not predict response to BCG treatment. Again, findings of this study could have been biased by its heterogeneous population, as it included tumours of different stage (Ta, T1, CIS) and grade (G1-G3) and patients having received different adjuvant treatment (BCG 27 mg, BCG 81 mg, BCG 27 mg + interferon alpha).

The novelty and strength of the present study therefore are in testing the predictive value of these markers in a homogeneous population of T1G3 tumours having undergone standardised BCG treatment (induction cycle, restaging TURBT, maintenance). Such strict inclusion criteria inevitably made the sample size relatively small, but

we believe that a well-selected and homogeneous population provides more valuable information than a larger but non-homogeneous one. Moreover, statistical significance despite the relatively small sample size should itself speak for the relevance of the findings.

We therefore believe that our results could be relevant when counselling patients with T1G3 BC about treatment options. Patients with wild-type or with altered expression of only one marker can be safely offered BCG treatment, as no such patient progressed in this study. Conversely, patients with altered expression of both markers should be informed of their 50% risk of tumour progression and, consequently, of potential advantages of early cystectomy (17).

Our findings also set the basis for searching for other markers whose altered expression would bring progression risk closer to 100%. Shariat *et al.* (16) have already shown that the higher the number of altered markers, the greater the risk of progression of non muscle-invasive bladder tumours. Prospective validation of altered expression of multiple markers in predicting the outcome of homogeneous populations of T1G3 bladder tumours treated with BCG is strongly needed to address the management of this challenging disease.

This is the first study demonstrating, in a well-selected and homogeneous population of T1G3 tumours, that altered p53 and pRb expression are predictive of response to BCG treatment. These findings can be valuable in counselling patients with this challenging disease. They also provide grounds for prospective validation of these and possibly other markers in risk stratification and management of newly diagnosed patients with T1G3 bladder tumours.

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