# Superficial papillary urothelial neoplasms of the bladder (PTA E PT1): correlation of expression of P53, KI-67 and CK20 with histologic grade, recurrence and tumor progression

## *Neoplasias uroteliais papilíferas superficiais da bexiga (pTa e pT1): correlação da expressão do p53, KI-67 E CK20 com grau histológico, recidiva e progressão tumoral*

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A B S T R A C T

**Objective**: To investigate the immunohistochemical expressions of p53, ki67, CK20 in superficial papillary urothelial neoplasms of the bladder and correlate them with histologic grade, tumor progression and recurrence. **Methods**: We selected samples of 43 patients with superficial transitional cell carcinoma of the bladder. They were divided into two groups, one called Recurrent (R), with 18 individuals, and other Non-Recurrent (NR), with 25. Multi-sampling blocks were prepared. The immunohistochemical technique employed was immunoperoxidase, and the antibodies were: p53: Novocastra (clone DO7) at a dilution of 1/100; Ki67: Spring (clone SP6) at a dilution of 1/100; and CK20: Dako (clone K20 .8) at a dilution of 1/50. **Results**: The expression of p53 was observed in 11 cases, six in the Recurrent group and five in the Non-Recurrent, all high-grade tumors (p = 0.0001). The histologic progression occurred in six patients (p = 0.0076). Of the 18 Recurrent cases, six showed immunoreactivity for p53 and 12 were negative for this antibody (p = 0.1715). Ki67 was positive in 17 of the 18 cases from the Recurrent group (p = 0.0001) and, from 20 high-grade tumors, 18 showed reaction to this antibody (p = 0.0001). Of the 18 individuals who had recurrence, 13 showed anomalous expression for CK20 (p = 0.0166). In high-grade carcinomas, of the 20 cases, 16 showed anomalous expression for this antibody, while 18 of the 23 patients with low-grade tumors showed normal expression for CK20 (p = 0.0002). **Conclusion**: The p53 showed good correlation with histologic progression and histologic grade. Ki67 was strongly associated with recurrence and histologic grade, and CK20 was also associated with these variables.

**Key words**: Urinary bladder. Neoplasms. Urinary bladder neoplasms. Immunohistochemistry. Biological makers.

## INTRODUCTION

Transitional cell carcinomas (TCC) correspond to 90% of all cases of malignant tumors of the bladder, most often involving male patients (2.5:1).Their incidence increases with age. This type is the second most common cancer of the genitourinary tract, after prostate cancer. It is the fourth most common among men and eighth among women. Some risk factors are involved in the carcinogenesis, such as smoking, calculi, chronic infections, urinary tract instrumentation and occupational exposure to petroleum, dye and rubber[1].

At diagnosis, they can present as advanced disease, invading the detrusor muscle layer, or as limited disease, confined to mucosa or lamina propria, also known as superficial or non-muscle-invasive tumors. This latter form is the most common, seen in approximately 75% of cases. Carcinomas "in situ" may also be considered non-muscle-invasive; however, they tend to be more aggressive and are often associated with high-grade TCC[2,3]..

Superficial bladder tumors can be managed with trans-urethral resection. This type of procedure is both diagnostic and therapeutic, as it allows direct visualization of the tumor, permits its removal and adjacent mucosal biopsies, aiming to exclude association with carcinoma "in situ"[4]..

Depending on the characteristics of the patients, the probability of recurrence one year after the transurethral

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resection may vary from 15% to 70%[5] and the likelihood of progression in five years varies from 7% to 40%[6]. Chemotherapy and / or immunotherapy can be used intravesically in superficial tumors, treating persistent microscopic tumors, preventing neoplastic reimplantation, tumor neoformation and possibly histologic and clinical progression[4,7]. However, not all of these neoplasms have the same risk of recurrence and progression. The absence of a risk stratification between the more aggressive tumors and the indolent one, eventually results in excessive monitoring or unnecessary intravesical therapy[8].

Prognostic factors in patients with superficial papillary urothelial cancer of the bladder have been the subject of publications in recent years[5,6,9,10,11]. Clinical and morphological parameters (size, grade, multifocality and carcinoma "in situ" in the adjacent mucosa) have shown inefficient and possibly increase the sensitivity and specificity, leading to different clinical management. So, recent research has been conducted correlating the involvement of p53, a tumor suppressor gene, ki67, a marker of cell proliferation and cytokeratin (CK20), with the histologic grade, progression, clinical staging and recurrence of bladder tumors[12,17].

Despite the efforts to show the importance of immunohistochemistry in risk stratification of papillary urothelial neoplasias of the bladder, little is done in the routine use of biomarkers. In one study in 335 laboratories in 15 countries, pathologists reported immunohistochemistry as being used by 91.7% of cases as an aid in the diagnosis of bladder cancer. Among the most commonly used markers are CK20 (76.9%), CK 7 (66.7%) and ki67 (38.8%). Only 24.8% reported the use of ki67 (84.4%) and p53 (64.4%) as prognostic indicators[18].

As there has been demonstrated that bladder tumors have different biological behaviors, efforts are being made in order to stratify them molecularly. The purpose is to define cancers with unfavorable outcome and to reconsider the current treatment for superficial tumors diagnosed only morphologically[12-17,19].

The objective of this study was to correlate the immunohistochemical expression of p53, Ki67 and CK20 in superficial papillary urothelial neoplasms of the bladder with prognostic factors of recurrence, tumor progression and histologic grade.

## METHODS

After approval by the Ethics Committee of the University of the Itajai Valley, (UNIVALI), Santa Catarina State – SC, Brazil, under registration 04/11b, we selected 59 patients who underwent transurethral resection due to bladder cancer during the period between January 2005 and December 2010 in the Clinic of Urology, UNIVALI. Inclusion criteria were: 1) patients with an initial diagnosis of superficial TCC of the bladder who underwent complete transurethral resection, or with new transurethral resection in 30 to 45 days, according to the protocol used by the Cancer Care Unit (UNACON); 2) patients with diagnosis of superficial bladder TCC, showing recurrence(s), regardless of histologic grade and stage; 3) possibility of access to all paraffin blocks required for the study. Exclusion criteria were: 1) patients who did not meet the inclusion criteria; 2) patients with multifocal tumors; 3) patients with carcinoma "in situ" in the adjacent mucosa or other histologic types or non-papillary urothelial tumors; 4) patients with previous chemotherapy or radiotherapy.

With the purpose of minimizing costs, we prepared multi-sample blocks, containing multiple tissue samples. These blocks were cut with a thickness of 4 ìm and submitted to routine staining for p53, Ki67 and CK20.

### Immunohistochemistry

It was performed according to the immunoperoxidase technique. The antibodies used were p53 (clone DO7; dilution 1/100), ki67 (clone SP6; dilution 1/100) and CK20 (clone K20.8; dilution 1/50), all monoclonal antibodies.

Incubation of the primary antibodies was performed during the overnight, period and kept at 5° C. After this period, the secondary antibody linked to the dextran polymer was incubated with the material for 30 minutes at room temperature. The DAB complex was then added to the slides for three minutes, according to conventional techniques. Counter-staining was made with Mayer's haematoxylin for one minute for CK20 and 30 seconds for the Ki67 and p53. Then dehydration was carried out with baths of 100% ethanol and clarification with xylene at room temperature. To mount the slides we used the Canada balsam technique, similar to the routine.

As for the interpretation of antibodies p53 and Ki67, cells were deemed positive if nuclear immunoreactivity reached 10% and 20%, respectively. The CK20 was considered to have anomalous expression when all layers of the epithelium were immunostained with standard plasma membrane, or when no cell showed antibody reaction. External controls were used, all known to be positive for antibodies used in this study. For p53, Ki67 and CK20, were respectively used samples of breast carcinoma, hyperplastic germinal centers of follicles of appendix and fragments of colonic mucosa. All tissues serving as positive controls were obtained from samples of radical surgical specimens.

### Statistic analysis

The nonparametric Fisher test was used to assess the correlation between histologic grade, tumor recurrence and progression with the expressions of p53, Ki67 and CK20 antibodies. The correlation between variables was significant at $p < 0.05$.

## RESULTS

Of the 59 patients who underwent bladder transurethral resection, 43 met the inclusion criteria and were divided into two groups: the first was composed of 25 (58%) patients with no recurrence, and the second consisted of 18 (42%) patients that had at least one recurrence during 12 to 71 months. Their ages ranged from 39-85 years (mean 66.58, median 70), and 35 (81.4%) were men and eight (18.6%) women. The gender distribution between groups revealed that, in non-recurrent cases, 19 (76%) patients were men and six (24%) women. In the recurrent group, 16 (88.8%) were men and two (11.2%) women.

In the group of non-recurrent tumors (25 cases), 16 (64%) were low-grade TCC and nine (36%) high-grade. Among the group of recurrent tumors, two cases initially diagnosed as Papillary Urothelial Neoplasms of Low Malignant Potential – PUNLMP – were reclassified as low-grade TCC. Thus, in this group of 18 cases, 13 (72.2%) were low-grade TCC and five (27.8%) of high grade. Statistical analysis showed no difference between the two groups (p = 0.2233).

Of the 18 recurrence cases, 14 (77.7%) had only one relapse. Of these, five were low-grade TCC and nine high-grade. Of these, five had low-grade tumors (progression). Only one case (5.5%) had two relapses, both low-grade. Finally, three cases (16.7%) with initial low-grade tumor developed recurrences, one having three recurrences, all high-grade. Another case presented with a low-grade tumor since the beginning as maintained this grade in the following three recurrences. The last patient had initially a high-grade tumor, and also on the three recurrences.

The pathological staging in initial tumors in the group of non-recurring tumors showed that 24 (96%) were limited to the mucosa (pTa) and only one (4%) infiltrated the lamina propria (pT1). In the group of recurrent tumors 17 (94.4%) were pTa and only one (5.6%) pT1 (p = 0.4983). Statistical analysis showed no statistical difference between the two groups.

Tumor size of non-recurrent tumors ranged from 0.8cm to 4.5 cm and 0.6cm to 8.7cm in the recurrent. The sizes were converted into qualitative variables, with cutoff in 3.0cm. So, from the 25 cases of the non-recurrent group, 13 had more than 3.0cm and 12 less. In the recurrent group, of the 18 subjects, 11 had dimension greater than 3.0cm and seven less than 3.0cm (Table 1).

Regarding progression of pathological stage / histologic grade, from the 18 recurrent cases, 14 had only one relapse. Of these, five (35.7%) progressed histologically and four (22.2%) in staging. In the five remaining cases, no tumor progression was found. Only one patient had two recurrences, without, however, exhibiting tumor progression. Three patients had three relapses. Of these, two progressed, one of them histologically, one in staging and one maintained the same grade and stage.

### Immunohistochemistry

The expression of p53 was observed in 11 (25.6%) cases, six (54.5%) from the recurrent group and five (45.5%) from the non-recurrent. In cases that progressed histologically, six were marked with this antibody. Nevertheless, there were mutations in p53 in all situations in which there was no histologic progression. The positivity for this antibody happened only in one case of staging progression. All high degree TCCs showed immunostaining for p53 and no low-grade TCCs did.

Ki67 positivity was observed in 25 (58.1%) cases, eight (32%) recurrent and 17 (68%) not. Only one case in the recurrent group was negative for this antibody. Progressions in histologic grade and staging were observed, respectively, in six (35.3%) and five (29.4%) cases, all positive for Ki67. Regarding histologic grade, high-grade TCCs had a higher frequency of positivity for this antibody, 18 (90%) cases. In only two (10%) high-grade tumors Ki67 was negative.

As for CK20 in the non-recurrent group, abnormal expression was shown in nine (36%) of the 25 cases. In the recurrent group, this same change was observed in 13 (72.2%) of the 18 cases. When there was histologic progression, four cases showed changes in the expression of CK20. The same frequency was found in cases where the histologic progression did not occur. Among patients

**Table 1-** Distribution of clinical stage, histologic grade and tumor size between groups.

| | Non-recurrent tumors | Recurrent tumors | |
|---|---|---|---|
| pTa | 24 | 17 | |
| pT1 | 1 | 1 | |
| | | | (p=0.4983) |
| High-grade | 9 | 5 | |
| Low-grade | 16 | 13 | |
| | | | (p=0.2233) |
| <3.0cm | 13 | 11 | |
| >3.0cm | 12 | 7 | |
| | | | (p=0.2067) |

who progressed in stages, five showed aberrant expression for this antibody. This same situation was observed in four cases in which there had been no progression in stage. Of the 14 tumors classified as high-grade, 12 (85.7%) showed aberrant expression of CK20 and two (14.3%) did not. Among the low-grade TCCs, the proportion of cases with anomalous expression for this antibody and those without it were, respectively, 10 (34.5%) and 19 (65.5%) cases (Table 2). In all cases with aberrant expression of CK20, there was diffuse positivity (Figure 1). No case showed absence of immunoreaction.

## DISCUSSION

According to the latest global estimates, in 2008 there were approximately 386,000 new cases of bladder cancer and 150,000 deaths from this neoplasm. The higher Brazilian incidence of this tumor is in Porto Alegre, capital of Rio Grande do Sul State, South Region, with 15.6 / 100,000 men. It is estimated that in 2012 6,210 new cases will arise in men and 2,690 in women. In Santa Catarina, another southern State, the estimated incidence of bladder cancer for 2012 is 8.02/100,000 men and 2.24 / 100,000 women[20].

TCC affects predominantly males, with male / female ratio of 3.16 / 1. In the recurrent group, even more so, 8/1. In this study, the mean age was 66.58 years, being very similar to those observed in other studies.

Approximately 80% of bladder cancers are superficial and 70% recur after resection[13]. Multifocality is also relatively common in these neoplasms[21], and its presence is one of the risk factors associated with recurrence[22]. According to Garcia Rodriguez *et al.* [17], women had a higher risk of multifocality and recurrence in six months. In this paper, the low number of women was not statistically sufficient for such correlation.

**Table 2 -** Distribution of p53, Ki67 and CK20 in relation to tumor progression, recurrence and histologic grade.

| | | p53+ | P53- | Ki-67+ | Ki-67- | CK20* | CK20 |
|---|---|---|---|---|---|---|---|
| Tumor progression | With histologic progression | 6 | 5 | 6 | 0 | 4 | 2 |
| | No histologic progression | 0 | 7 | 6 | 1 | 4 | 3 |
| | p value | p=0.0076 | | p=0.5385 | | p=0.4079 | |
| | With clinical progression | 1 | 4 | 5 | 0 | 5 | 0 |
| | No clinical progression | 1 | 7 | 6 | 1 | 4 | 3 |
| | p value | p=0.5128 | | p=0.5833 | | p=0.1591 | |
| Relapse | Present | 6 | 12 | 17 | 1 | 13 | 5 |
| | Absent | 5 | 20 | 8 | 17 | 9 | 16 |
| | p value | p=0.1715 | | p=0.0001 | | p=0.0166 | |
| Histologic grade | High-grade | 11 | 9 | 18 | 2 | 16 | 4 |
| | Low-grade | 0 | 23 | 7 | 16 | 5 | 18 |
| | p value | p=0.0001 | | p=0.0001 | | p=0.0002 | |

*+ nuclear immunoreactivity in neoplastic cells exceeding 10% and 20%, respectively for p53 and Ki67; * anomalous expression.*

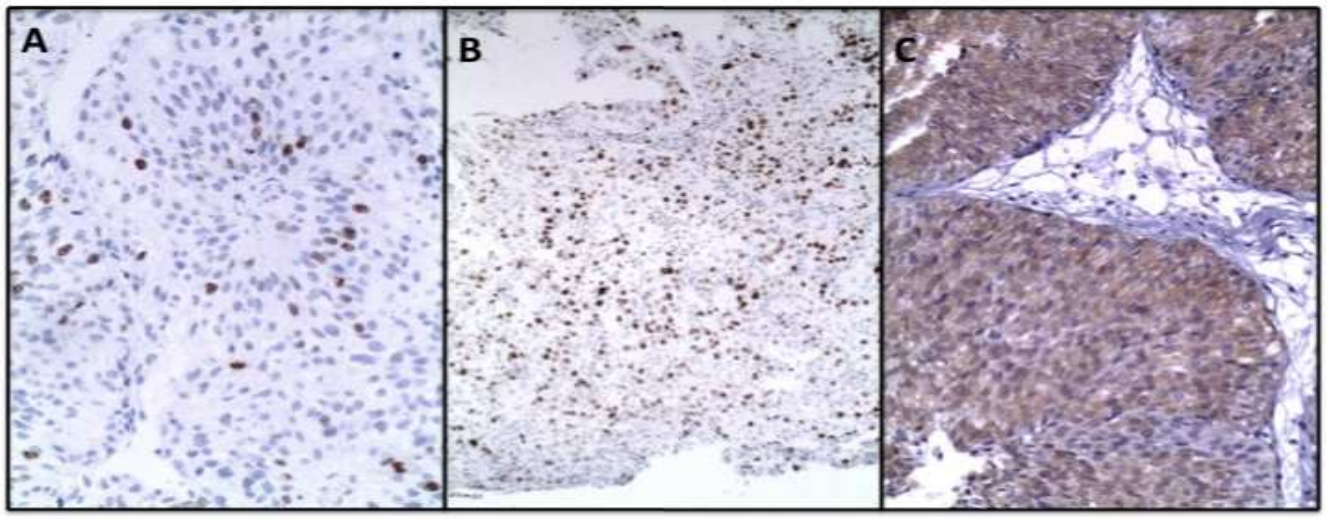


***Figure 1 -*** *Photomicrograph (400X) showing, in A and B, nuclear positivity for Ki67 and p53, respectively; in C, aberrant expression of CK20 in the entire urothelium mass.*

Pathological staging is an important predictor of recurrence and especially tumor progression[23,24]. To Lee *et al*. [24], recurrence was not associated with early stage tumors. However, the increase was greater in those who had the initial lesion with deep invasion of the lamina propria. In this study, this comparison was not possible because there was only one pT1 case in each group.

**Papillary urothelial neoplasms biomarkers**

Biomarkers are substances that objectively measure or evaluate biological processes and pathological or pharmacological responses to therapeutic intervention[25]. Few studies have been conducted involving immunohistochemical markers – bcl-2, p53, ki67 and CK20 – with classic prognostic factors and recurrence. However, the results are contradictory and the series are difficult to be compare due to heterogeneity in different clinical stages[13]. Theoretically, these biomarkers could be an alternative to stratify TCC patients in different risk groups.

P53 has been associated with prognostic parameters such as recurrence, tumor progression and metastasis-free interval. The expression of this antibody was associated with tumor progression in some studies[3,12,14,19]. Unlike the results of many others, Moyano Calvo *et al* [26] did not observe the association between p53 and progression, due to low rate of antibody positivity. This study revealed data very close to the international literature, showing a strong association of p53 with histologic progression (p = 0.0076). The histologic grade has also been related to p53 since studies have shown increased expression of this marker in higher grade tumors[12,15,27]. In this study this factor was also observed (p = 0.0001). Here, p53 was not correlated with recurrent cases (p = 0.1715). In literature this association showed contradictory results. While for some authors, p53 was associated with recurrence[13,22], for others there was no statistical significance in this regard[12,19].

The expression of Ki67 in the urothelium is low and limited to basal layers. Authors agree that it varies considerably with histologic grade and clinical stage[3,13,16,26,28]. In this study, the expression of Ki67 was significantly higher in high-grade tumors (p = 0.0001), as literature says. Finally, this series found a significant association of ki67 and recurrence (p = 0.0001). Nevertheless, this correlation also shows conflicting results in different studies. According to Rodríguez-Alonso *et al* , the correlation of relapse with ki67 was not significant in one of their papers[14]. However, these same authors conducted a subsequent study that showed statistical significance between relapse and ki67, when the expression of this antibody was superior to 27%[22]. By analyzing the results of these researchers, it can be seen that in the research done in 2002, where ki67 was not correlated with relapse, most of the samples consisted of high-grade tumors. The literature shows that high-grade TCCs present more often with high proliferative indices[3,13,15,26,29]. So, one of the reasons for the lack of association in this study between Ki67 and recurrence is a selection bias of sampling. That is because there is no acceptable proportion of low-grade tumors to establish a correlation between these two variables. In other studies, this discrepancy in results persists. While for some authors, there is an association of ki67 with recurrence[16,26,28], for others it remains fragile[13,29]. Perhaps this difference may be a result of different classification systems, cutoffs employed and different monoclonal antibodies.

In the urothelium, the CK20 is normally expressed only in the cells surface. Some researchers have reported the dedifferentiation of CK20 in recurrent disease, especially pTa/pT1 TCCs[15,30-32]. In contrast, many studies have also shown mixed or inconsistent results[3,13,28,33]. Regarding the anomalous expression of CK20, these results showed statistical association with recurrence (p = 0.0166) and histologic grade (p = 0.0002). Some published studies conclude that aberrant expression of CK20 may be useful in distinguishing papillary urothelial neoplasms of low malignant potential from low-grade TCCs. Furthermore, the differences between low and high-grade TCCs were statistically significant[15,30,33]. These data reflect the results of this research.

The use of immunohistochemical markers, based on research data, may be useful as additional criteria in risk stratification of patients with non-invasive TCC. However, given the different findings in the literature, it is necessary to continue the studies to deepen the knowledge related to the use of biomarkers in patients with bladder cancer.

In conclusion, p53 showed good correlation with progression and histologic grade. Ki67 is strongly associated with relapse and histologic grade and CK20 was also associated with these variables.

## RESUMO

***Objetivo:*** *Investigar a expressão imunoistoquímica dos marcadores p53, Ki-67, CK20 em neoplasias uroteliais papilíferas superficiais da bexiga e correlacionar com o grau histológico, progressão tumoral e recidiva.* ***Métodos:*** *Foram selecionadas amostras de 43 pacientes portadores de carcinoma de células transicionais superficiais da bexiga. Elas foram distribuídas em dois grupos, um denominado recorrente, de 18 indivíduos e outro não recorrente, com 25 casos. Foram confeccionados blocos multiamostrais. A técnica imunoistoquímica empregada foi de imunoperoxidase e os anticorpos foram: p53 (clone DO7), o Ki-67 (clone SP6) e CK20.* ***Resultados:*** *A expressão do p53 foi observada em 11 casos, todos tumores de alto grau (p=0,0001). A progressão histológica ocorreu em seis indivíduos (p=0,0076). Dos 18 casos recorrentes, seis apresentaram imunorreação para o p53 e 12 foram negativos para este anticorpo (p=0,1715). O Ki-67 foi positivo em 17 dos 18 casos do grupo recorrente (p=0,0001) e dos 20 tumores de alto*

*grau, 18 apresentaram reação para este anticorpo (p=0,0001). Dos 18 indivíduos que tiveram recorrência, 13 apresentaram expressão anômala para CK20 (p=0,0166). Nos carcinomas de alto grau, dos 20 casos, 16 apresentaram expressão anômala para este anticorpo, enquanto que 18 dos 23 indivíduos com tumores de baixo grau mostraram expressão habitual para a CK20 (p=0,0002).* ***Conclusão:*** *O p53 mostrou boa correlação com a progressão histológica e grau histológico. O Ki-67 apresentou forte associação com a recidiva e grau histológico, e a CK20 também associou-se com estas variáveis.*

***Descritores:*** *Bexiga urinária. Neoplasias. Neoplasias da bexiga urinária. Imunoistoquímica. Marcadores biológicos.*

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