

# Cytokeratin 20, 34 $\beta$ E12 and overexpression of HER-2/neu in urine cytology as predictors of recurrences in superficial urothelial carcinoma

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## Abstract

There were investigated 80 patients with superficial urothelial carcinoma of the urinary bladder (Ta and T1). All cases had a minimum follow-up of 36 months. Patients were selected in order to form two subgroups. The first group (n = 28) had no recurrence during the follow-up, and in the second (n = 52) each patient had a minimum of two recurrences. The pathologic diagnosis was performed on archive slides stained with haematoxylin-eosin. Archive smears performed at the moment of the primary diagnosis from voided urine were discolored, and used for immunocytochemistry. It was investigated the expression of cytokeratin 20, high molecular weight cytokeratin 34 $\beta$ E12, and overexpression of HER-2/neu protein. Microwave antigen retrieval was performed in all immunocytochemical procedures. The working system was LSAB2 and EnVision, and diaminobenzidine was used as chromogen. The initial conventional cytology was positive for malignant cells in all cases. Cytokeratin 20 was positive in 92.85% of cases without recurrences, and in 94.23% of cases with recurrences. High molecular weight cytokeratin was expressed in 42.85% of cases without recurrence and in 63.46% of patients with recurrences. The overexpression of HER-2/neu protein was found in 7.14% of cases of the first group, and in 84.61% of cases of the second group. Our results support the idea that HER-2/neu and high molecular weight cytokeratin may be used as markers to predict recurrence in superficial urothelial carcinoma.

**Keywords:** urothelial carcinoma, recurrences, cytology, immunocytochemistry, cytokeratin, HER-2/neu protein.

## Introduction

Urothelial carcinoma is the most frequent malignant tumor of the urinary bladder and occupies the fifth place between human malignancies [1]. About 60% to 65% of urothelial carcinomas are superficial in terms of the clinical classification, and include Ta and T1 tumors that usually are surgically treated by transurethral resection. On the other hand, recurrences are noticed in almost 70% of them within 24 months in the absence of adjuvant therapy. Moreover, many cases have multiple recurrences that imply repeated surgical treatment with consecutive well-known complications. Because of these reasons, many researchers focused their efforts to identify some markers with predictive value of recurrences even from the primary diagnosis [2, 3].

The nuclear markers of cell proliferation were extensively investigated, but results showed correlation with the degree of differentiation and therefore, they could be not considered independent factors useful to predict recurrences [4–6].

The immunocytochemical expression of the normal and tumor urothelium specific-cytokeratin was investigated by a reduced number of authors [7]. Results published until now on this topic are controversial, despite of the fact that some reports suggested the predictive value for recurrences of cytokeratin 20 [8].

The expression of cytokeratin 7 seems not to be helpful to predict recurrences, and, to our knowledge, the value of high molecular weight cytokeratin was not yet investigated. Taking into account that cytokeratin are intermediate filaments found in the cytoplasm of epithelial cells, we associated in the study the

overexpression of the HER-2/neu protein that is found at the plasma membrane level and reflects an active proliferation potential.

HER-2/neu gene encodes the transmembrane tyrosin-kinase growth factor. Nowadays, it is thought that this receptor is involved in the control of the cell growth and differentiation. Overexpression of this protein correlates with an unfavorable prognosis in breast and ovarian carcinoma, but at the same time, it represents an indication for the specific treatment with Trastuzumab.

The humanized monoclonal antibody (also known as p185) was demonstrated to be efficient in these tumors, as monotherapy and in combination with Cisplatin. The expression of HER-2/neu in urothelial carcinoma was signaled out more than ten years ago [9].

Despite the distribution of the final product of reaction in well characterized, until now there is a lack of data about the relationship with prognosis and recurrences [10, 11].

Some studies demonstrated a correlation between the overexpression of HER-2/neu and unfavorable prognosis, but only in patients with invasive tumors. On the other hand, it was not found a relationship between overexpression and stage of the tumor and the status of lymph nodes [12].

A single publication showed a reduced expression of HER-2/neu in superficial urothelial carcinoma that correlates with a reduced rate of recurrences. Our purpose was to investigate the predictive value for recurrences of the immunocytochemical expression of cytokeratin 20, high molecular weight cytokeratin, and HER-2/neu.

## Material and methods

There were investigated 80 patients with superficial urothelial carcinoma. The primary cytodiagnosis performed on routine stained smears was positive for malignant cells in all cases.

The pathologic stage was Ta in 48 cases and T1 in 32 cases. The degree of differentiation, G, was established according Mostofi system, and there were 34 G1 cases, 29 G2, and 17 G3 cases.

Transurethral resection was performed in all patients, and all had a minimum follow-up of 36 months. After the follow-up patients were divided in two subgroups: 28 patients without recurrence within 36 months, and 52 with two or more recurrences. Archive smears from these patients were immunocytochemically stained for cytokeratin 20 (clone), high molecular cytokeratin (clone 34 $\beta$ E12), and HER-2/neu protein.

The working system was labeled streptavidin biotin (LSAB2) to demonstrate cytokeratin, and EnVision for HER-2/neu protein. The final product of reaction was visualized with diaminobenzidine and nuclei were stained with Lillie's modified Haematoxylin. All reagents for the immunocytochemical procedure were from DakoCytomation (Denmark).

The immunoreaction for cytokeratin 20 and high-molecular weight cytokeratin was considered positive when minimum 10% of malignant cells were stained with diffuse, cytoplasmic pattern. Interpretation of HER-2/neu stained smears was based on the system largely accepted in breast carcinoma and recommended by the manufacturer (0 and +1 negative, and +2 and +3 positive). Only cells with intense positive reaction at the level of the plasma membrane were considered to be positive. The statistical analysis was performed with commercially available SPSS10.0.

## Results and discussions

The immunoreaction for cytokeratin 20 was positive in 26 (92.85%) from 28 cases without further recurrences, and in 49 (94.23%) from 52 cases with recurrences. The statistic analysis showed no significant difference between the two subgroups ( $p < 0.1$ ).

The final product of reaction for cytokeratin 20 was restricted to the cytoplasm, it was intensely stained, with diffuse pattern, and occasionally, vacuolated in the paranuclear space (Figure 1). In the cases with positive results, almost all malignant cells were intensely stained, even the case of cells with reduced amount of cytoplasm.

High molecular weight cytokeratin was positive in 12 (42.85%) from 28 cases of the first group, and in 33 (63.46%) from 52 cases of the second.

Results were statistically significant for  $p < 0.001$  (standard deviation 3.53). The immunoreaction was strong or moderate as intensity, always with homogeneous cytoplasmic pattern. The same pattern of distribution was noticed in isolated and clusters of malignant cell (Figure 2, a and b).

The overexpression of HER-2/neu protein was interpreted according general accepted criteria for breast

carcinoma. The immunoreaction was quoted with "0" if any positive reaction can be observed in the plasma membrane (Figure 3a). The reaction was noted with "+1" if the reaction was positive at the membrane level, but with discontinuous pattern and in less than 10% of malignant cells (Figure 3b).

The reaction was classified as "+2" or "+3" (both positive) when the final reaction product was restricted at the plasma membrane, with continuous pattern and in more than 10% of malignant cells (Figures 4 and 5).

Overexpression of HER-2/neu protein was noticed in only 2 (7.14%) from 28 cases without recurrences, and in 44 (84.61%) from 52 cases with recurrences. The statistical analysis showed a significant difference for  $p < 0.0001$  (standard deviation 2.69, confidence interval 95%).

The overexpression was found to be more frequent in moderate and less differentiated urothelial carcinomas, and only rarely in well differentiated tumors. On the other hand, not all G3 tumors were positive (+2 or +3). The value of this three-immunocytochemical method to predict recurrences is shown in Figure 6, and curves are divergent for HER-2/neu only.

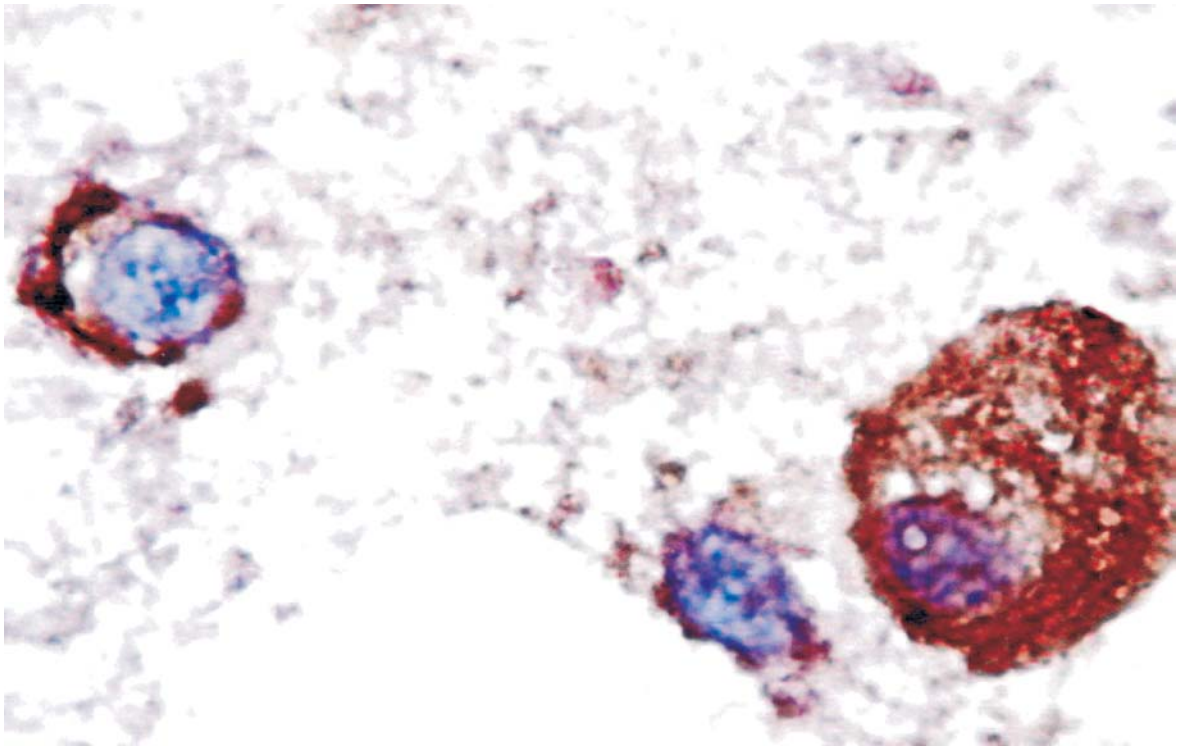
Urine cytology is thought to be "the gold standard" of the noninvasive diagnosis and follow-up of patients with bladder tumors [13, 14]. In the detection of recurrences, conventional cytology has better results than in the primary diagnosis, mainly in low-grade tumors. The sensitivity of the method in the diagnosis of recurrences is 93.8%, the sensitivity is 92.7%, and the predictive positive value is 78.9% [15].

In spite of its real diagnostic efficacy, urinary cytology was not demonstrated to be a good predictor of recurrences. Moreover, other biomarkers, like BTA stat, ImmunoCyt or human complement factor H related protein were not found to be useful in prediction of recurrences [16–19].

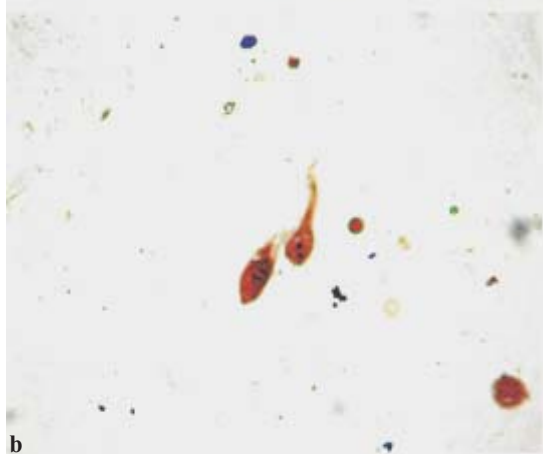
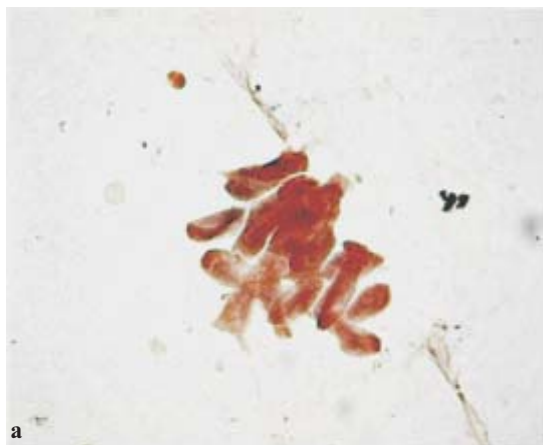
Only recently urinary cytology became a target for immunocytochemical methods. The immunohistochemical profile of normal and malignant urothelium is well known. The co-expression of cytokeratin 20 and cytokeratin 7 is a landmark of primary urothelial carcinoma and its metastasis as well. Few data are available about the expression of cytokeratin in cells exfoliated in the voided urine. Some author believe that such an expression of cytokeratin 20 on malignant urothelial cells may significantly decrease the rate of the so-called "atypical urothelial cells" [7, 20].

Ramos *et al.* (2003) noticed that expression of cytokeratin 20 in the primary tumor is a strong predictor for recurrence [8]. Our data do not support this finding, because it was found to be positive in 92.85% of patients without recurrence and in 94.23% of patients with recurrences.

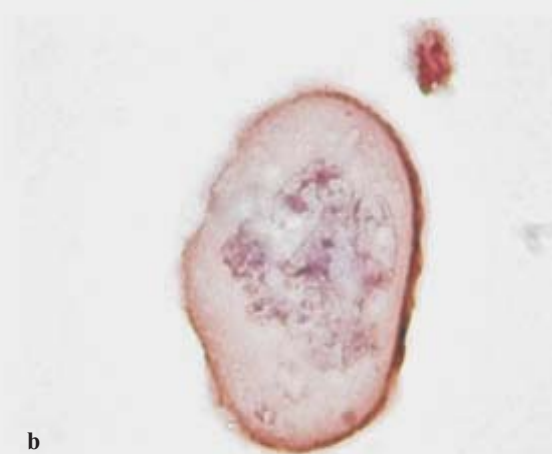
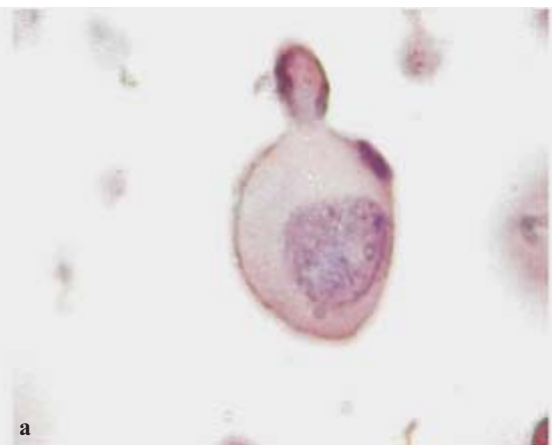
In our opinion, the expression of cytokeratin 20 reflects the urothelial differentiation of the tumor and cannot give any information about a further recurrence. To the best of our knowledge, in the literature was published a single article on the immunocytochemical expression of high molecular weight cytokeratin on urine cytology [8].



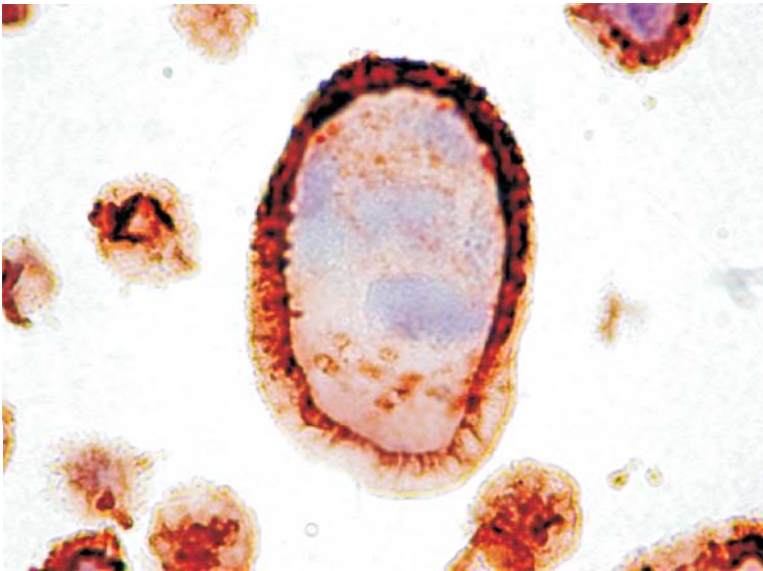
**Figure 1 – Ta G2 urothelial carcinoma. Immunocytochemical positive reaction for cytokeratin 20. Note the intense staining of the cytoplasm of malignant cells (Cytokeratin 20, EnVision, DAB,  $\times 1000$ )**



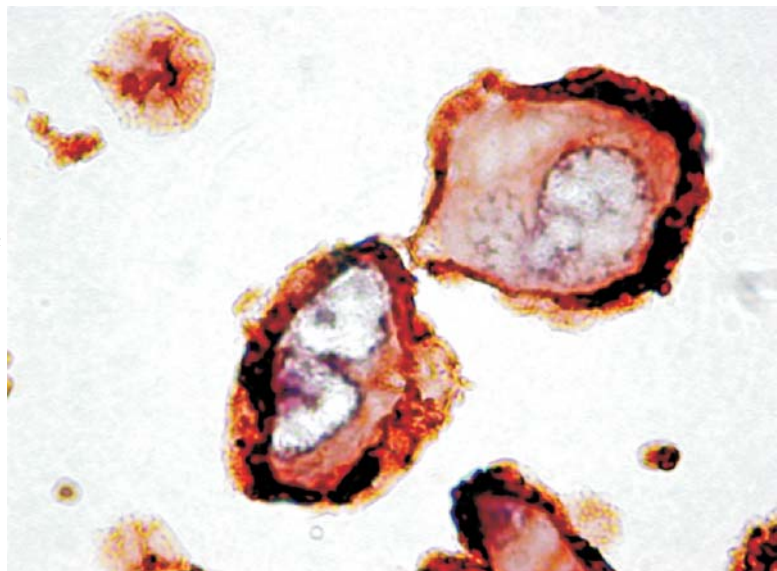
**Figure 2 – Recurrent Ta G1 urothelial carcinoma. Positive immunoreaction for high molecular weight cytokeratin (34 $\beta$ E12). Cluster (a), isolated cells (b) (EnVision, DAB,  $\times 200$ )**



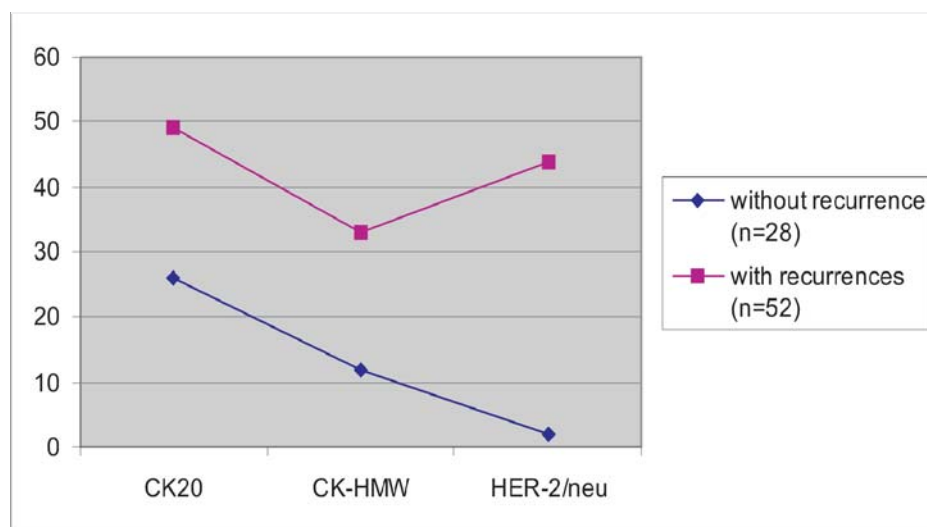
**Figure 3 – a) Moderate differentiated urothelial carcinoma negative for HER-2/neu protein; b) Anaplastic malignant cell with moderate plasma membrane-restricted, for HER-2/neu protein (EnVision, DAB,  $\times 1000$ )**



**Figure 4 – T1 G3 urothelial carcinoma. Anaplastic malignant cell, intensely positive for HER-2/neu protein (EnVision, DAB, ×1000)**



**Figure 5 – Large anaplastic malignant cell with intense immunoreaction for HER-2/neu at the plasma membrane level (EnVision, DAB, ×1000)**



**Figure 6 – Value of cytokeratin 20, high molecular weight cytokeratin, and HER-2/neu protein overexpression for prediction of recurrences in superficial urothelial carcinoma**

The authors cited above found a sensitivity of 75.6% and a specificity of 53.4% as predictive marker for recurrences. We found a positive reaction in 42.85% of patients without recurrences and in 63.46% of patients with recurrences. Results are significant for  $p < 0.001$ , but standard deviation was 3.53. Taking these data together, we think that this method deserves further investigations on large series.

The overexpression of HER-2/neu protein was investigated on paraffin sections from invasive urothelial carcinoma by many authors [12, 21, 22]. The overexpression correlates with the stage and grade of the tumor and it is thought to be a potential target for specific therapy. There is a lack of data regarding the application of this method in urinary cytology. We noticed positive reaction in only two cases without recurrences and in 44 cases with recurrences. This is why we believe that the overexpression of HER-2/neu protein could be the most useful method in prediction of recurrences of urothelial carcinoma.

### Conclusions

The immunocytochemical study of 80 patients with superficial tumors of the urinary bladder revealed the following aspects, regarding the value as predictors of recurrences: the expression of cytokeratin 20 reflects the urothelial differentiation of bladder carcinoma, but it has not value as predictor marker; the expression of the high molecular weight cytokeratin seems to be promising, but it needs to be studied on large series of patients; our results show that the overexpression of HER-2/neu protein in malignant cells from the voided urine has high predictive value for recurrences in superficial urothelial carcinoma.

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