

## P16, c-erbB2 and Ki67 immunoexpression in urothelial carcinomas of the bladder

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### Abstract

The study included a total of 28 cases of urothelial carcinomas, which were analyzed histopathologically and immunohistochemically using anti-human p16, c-erbB2 and Ki67 antibodies and the LSAB/HRP work system. Histopathological analysis revealed the presence of urothelial carcinomas with different degrees of differentiation and invasion in the lamina propria or muscularis propria. The immunostain for p16, c-erbB2 and Ki67 was present at the nuclear and cytoplasmic level, respectively at membrane and nuclear level. Immunoreactions had quantified values, which were statistically correlated with the degree of differentiation in case of c-erbB2 or depth of invasion for p16. Tumor activity status and the risk of progression of bladder urothelial carcinomas can be assessed objectively using the antibody panel consisting of p16, c-erbB2 and Ki67.

**Keywords:** urothelial carcinoma, p16, c-erbB2, Ki67.

### Introduction

Bladder urothelial carcinoma is a frequently diagnosis for the malignant tumor pathology in this location. The appearance and progression of these tumors involve biomolecular mechanisms supported by numerous proteins that may become therapeutic targets and are investigated in research programs. Tumor proliferation rate is the result of inactivation of protective factors and the occurrence of an imbalance in the cell cycle regulators. Antiproliferative effects of p16 are exerted in the G1 phase of the cell cycle by suppressing retinoblastoma protein phosphorylation, being a negative regulator [1, 2]. Also, p16 is part of a biomolecular system with cyclin D, PRB, CDK4 and 6, p53, components that influence the biomolecular activity and protein expression. However, p16 has been shown in some studies a viable biomarker for malignant superficial lesions of the urothelium [3, 4]. C-erbB2 (Her-2/neu) unlike p16 has opposite effect, stimulating cell proliferation. In contrast to mammary gland carcinoma, where the gene is amplified in most cases, for bladder urothelial carcinoma Her-2/neu is overexpressed in 10–50% of cases [5, 6]. Ki67 recognizes cells in the active phase of the cell cycle and in the case of bladder urothelial carcinomas has special significance in the sense of a suggestive prognostic assessment [7].

The aim of this study was to quantify the proliferation rate of bladder urothelial carcinomas in the context of its antagonistic effects of p16 and c-erbB2. We also followed the correlation between immunohistochemical expression of biomarkers and tumor grade and stage.

### Materials and Methods

The study included a total of 28 cases diagnosed

with bladder urothelial carcinoma, selected from the casuistry of Pathology Lab of the Emergency County Hospital of Craiova, in 2010. Biological material was represented by cystectomy pieces from patients hospitalized in the Urology Clinic of the same Hospital, which were processed by common histopathological technique, with paraffin embedding and Hematoxylin–Eosin stain. Each case was analyzed in terms of histological grade of differentiation and depth of invasion.

The immunohistochemical processing was made on serial sections using LSAB+ System-HRP (DAKO, code K0690) visualization system, the developing of reactions being made with DAB (3,3'-diaminobenzidine). We used monoclonal mouse antibodies anti-human p16 (diluted 1/100, clone DC-468, Santa Cruz Biotechnology), anti-human Ki67 (diluted 100, clone MIB1, DAKO), and rabbit polyclonal antibody anti-human c-erbB2 (diluted 1/250, DAKO). As antigen retrieval, we used Heat-Induced Epitope Retrieval (HIER) boiling the sections at microwave in citrate buffer pH 6 for p16 and c-erbB2 and in Tris-EDTA buffer pH 9 for Ki67. The counterstain of sections was done with Hematoxylin and also we used negative external control reactions.

P16 immunoreaction was considered positive if the stain was present at the cytoplasmic and/or nuclear level, being negative if the response was absent or focal, on small groups of cells [3, 8]; also was analyzed the intensity of reaction, characterized as strong or weak. Interpretation of c-erbB2 staining was done in accordance with the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria for breast carcinoma (score 0, no staining or membrane staining is observed in <10% of

the tumor cells; score 1+, faint/barely perceivable membrane staining is detected in >10% of the tumor cells, and only part of the membrane is stained; score 2+, weak to moderate complete membrane staining is observed in >10% of the tumor cells; score 3+, strong complete membrane staining is observed in >30% of the tumor cells) [9]. Also, the presence of cytoplasm stain was considered only when it was associated with a membranous stain. To quantify Ki67 immunoexpression a proliferation index (IP) was done by ratio of marked neoplastic cells and the total number of cells counted on 40× microscopic field, cutoff point being 10% [10].

To assess the dependence between the two classification factors, incidence tables were made which were interpreted using chi-square test, corrected with Yates index. The acquisition of the images was done with Nikon Eclipse E600 and software program Lucia 5.

## Results

Histopathological analysis of tumor lesions revealed the presence of well-differentiated carcinomas in nine cases, moderately differentiated in 11 cases and poorly differentiated in eight cases. Depending on the depth of invasion, most lesions were diagnosed with invasion in muscularis propria (T2a/b, 14 cases), followed by invasion of lamina propria and whole bladder wall

invasion (T1, 10 cases, respectively T3a four cases) (Table 1, Figure 1).

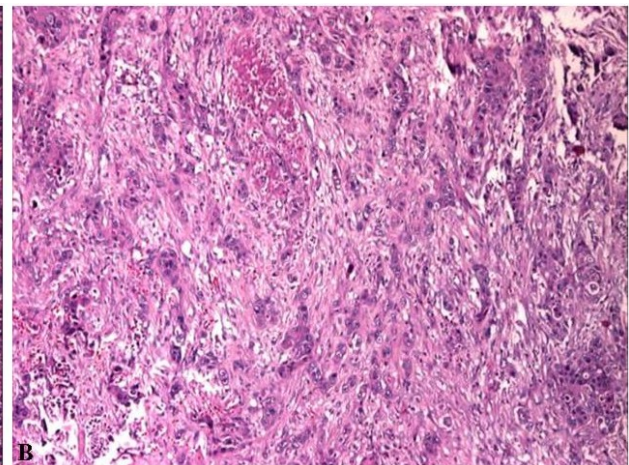
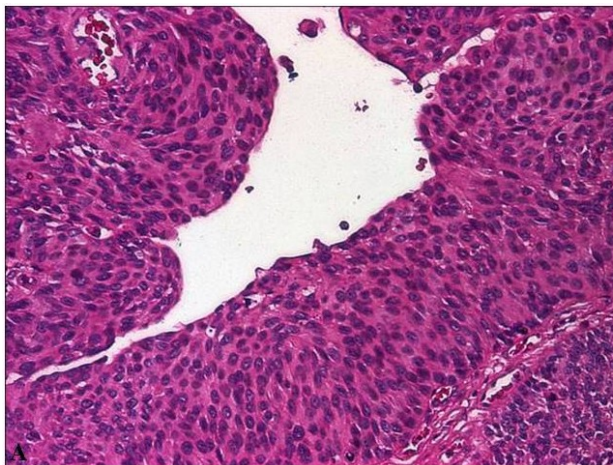
**Table 1 – Histopathological distribution of the cases**

Urothelial carcinomas	Well-differentiated	Moderate differentiated	Poorly differentiated
T1	8	2	–
T2a/b	1	7	6
T3	–	2	2

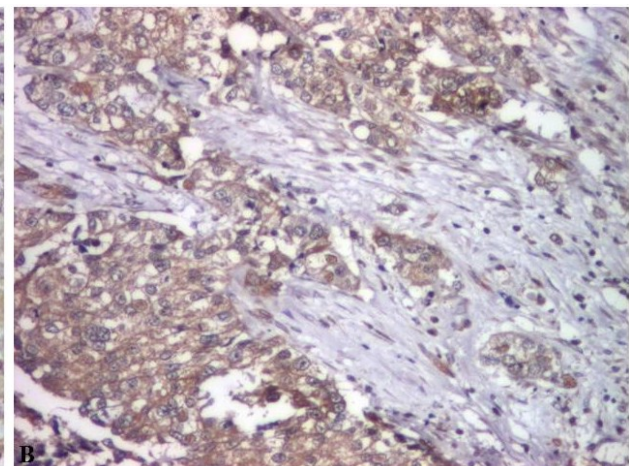
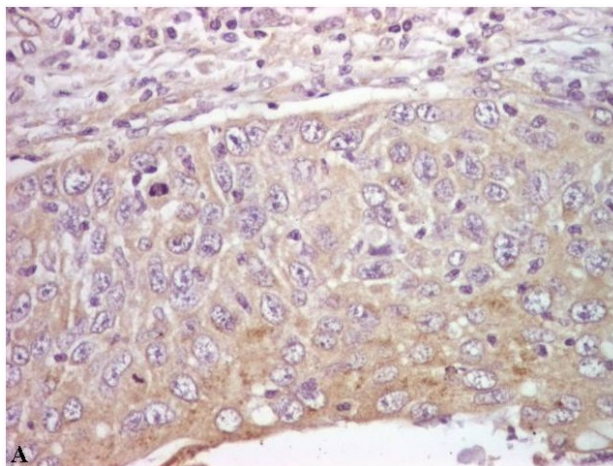
P16 immunoreactions were positive in 22 cases at the cytoplasmic and/or nuclear level. In case of low or moderate T3 differentiated carcinomas, the reaction was negative. The immunostain of low intensity was present only at the cytoplasmic level in six cases of well-differentiated T1 carcinomas. In seven cases of T1 or T2 carcinomas, well or moderately differentiated the stain was cytoplasmic, with low intensity and also we noticed some reactive nuclei. In nine cases of moderately or poorly differentiated T2 carcinomas, the immunostain was mixed nuclear and with high intensity (Table 2, Figure 2).

**Table 2 – P16 immunoexpression patterns**

P16 immunostain / Stage	T1	T2	T3
Cytoplasmic	6	–	–
Cytoplasmic or nuclear, low intensity	4	3	–
Cytoplasmic or nuclear, high intensity	–	9	–



**Figure 1 – (A) Well-differentiated urothelial carcinoma, HE stain, ×100. (B) Poorly differentiated urothelial carcinoma, HE stain, ×100.**



**Figure 2 – P16 immunostain, ×100. (A) Well-differentiated urothelial carcinoma, cytoplasmic pattern. (B) Poorly differentiated urothelial carcinoma with muscularis propria invasion, mixed cytoplasmic and nuclear pattern.**



In positive cases, have been identified rare stromal elements with positive nuclear reaction. Statistical analysis of the cases revealed a significant correlation of cytoplasmic/nuclear p16 immunostain with tumor stage (T1–T2) (Yates'  $p$ -value  $<0.001$ ) and no correlation with tumor grade (Yates'  $p$ -value  $=0.1$ ). There were no significant differences in the intensity of stain for different tumor grades and stages.

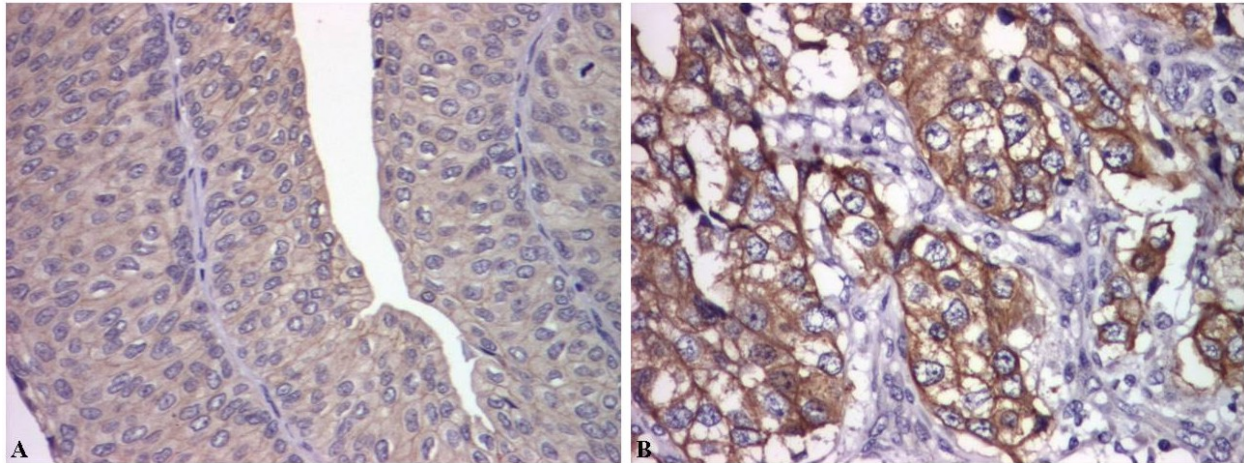
C-erbB2 immunohistochemical stain was identified at the membrane level in 19 cases. Negative reactions were present in nine urothelial carcinomas, which showed no particular morphological features, with varying degrees of differentiation and being on stage T1–T3. In five cases, the score was 1+, the stain was weak or incomplete on  $>10\%$  of the cells and tumors were well differentiated. In eight cases, the score was 2+, the stain being complete weak or moderate on  $>10\%$  of the cells and the tumors were moderately differentiated. In four cases and two cases of poorly respectively moderately differentiated carcinomas was 3+ score, the stain being intense and complete membranous in  $>30\%$  of cells (Table 3, Figure 3).

**Table 3 – C-erbB2 immunoexpression score**

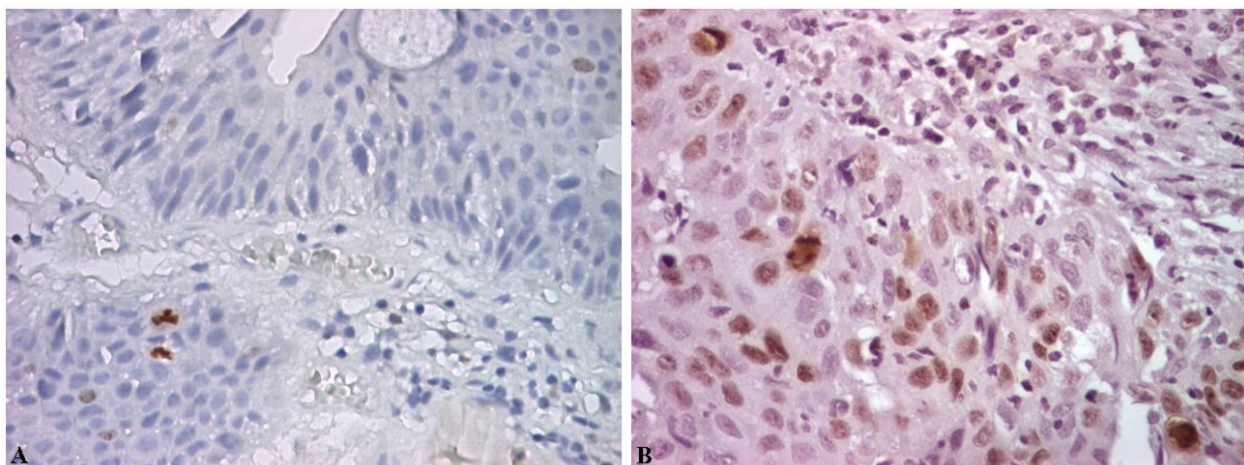
C-erbB2 immunostain / Grade	Well	Moderate	Poorly
1+	5	–	–
2+	–	8	–
3+	–	2	4

In particular, for well or moderately differentiated carcinomas, the membranous immunostain was accompanied by a diffuse cytoplasmic one. Statistical analysis of the cases showed a significant correlation of c-erbB2 immunoexpression and the degree of tumor differentiation (Yates'  $p$ -value  $=0.04$ ). We found no statistical correlation in terms of immunoexpression score and tumor stage (Yates'  $p$ -value  $=0.07$ ).

Ki67 immunoreaction was considered positive in 22 cases being present at the nuclear level. In six cases of well-differentiated carcinomas in stage T1, Ki67 proliferation index was  $<10\%$ . Proliferation index values ranged between 5% and 54%, the highest values being recorded for T3 moderately or poorly differentiated carcinomas (Figure 4).



**Figure 3 – C-erbB2 immunostain,  $\times 100$ . (A) Well-differentiated urothelial carcinoma, score 1+. (B) Poorly differentiated urothelial carcinoma with muscularis propria invasion, score 3+.**



**Figure 4 – Ki67 immunostain,  $\times 200$ . (A) Well-differentiated urothelial carcinoma,  $IP < 10\%$ . (B) Moderately differentiated urothelial carcinoma,  $IP = 52\%$ .**

Statistical analysis revealed significant correlation between Ki67 proliferation index, tumor grade (Yates'  $p$ -value  $<0.0001$ ) and tumor stage (Yates'  $p$ -value  $=0.01$ ).

Results for biomarkers using immunohistochemical reactions showed a direct correlation between Ki67 and p16 immunoexpression (Yates'  $p$ -value  $=0.02$ ) and between Ki67 and c-erbB2 (Yates'  $p$ -value  $=0.05$ ), with

no association between expression of p16 and c-erbB2 (Yates'  $p$ -value = 0.18).

## Discussion

In the literature data, there are numerous studies that want to demonstrate the prognostic value of some biomarkers involved in the biomolecular mechanisms of the initiation and progression of urothelial cancer process. Among these p16 and c-erbB2 have proven their practical usefulness in assessing the exocervix squamous intraepithelial lesions respectively for the breast cancer lesions [11, 12]. Regarding the urothelial carcinomas the studies realized until now provide conflicting data about the expression of p16 and c-erbB2 and meaning of this expression.

P16 is a negative regulator of cell cycle providing control cell of the transition from G1 phase to S phase [13]. During tumor development, mitogenic stimuli such as growth factors leading to activation of cyclin D, which binds and activates the cyclin-dependent kinases 4 and 6, resulting in retinoblastoma protein phosphorylation and issue a transcription factor that ensures cell proliferation [4]. P16 binds kinase complex and prevents phosphorylation, thereby having an anti-proliferative effect [14].

In this study, p16 immunoexpression was identified at the cytoplasmic and/or nuclear level in 78.5% of cases and it is correlated with tumor stage T1–T2, T3 lesions being negative.

Literature data revealed that protein is involved in the initial stages of carcinogenesis and tumor progression, with some studies showing protein overexpression at the tumoral invasion front [13, 15]. In a study conducted by Cui L *et al.* in 2006 [16], the authors concluded that p16 immunoexpression decreases in invasive urothelial carcinomas. Some authors have suggested that cytoplasmic expression of the marker in premalignant and invasive lesions can be the result of a counter of cyclin-dependent kinases, and others have supposed that such a staining is an expression of protein inactivation [3, 17].

In a study conducted in 2007 by Yin M *et al.* [3], on 80 cases of carcinoma *in situ* and invasive urothelial carcinomas, reported that the p16 immunoexpression was predominantly cytoplasmic with variable nuclear stain. He also found an increased protein expression for invasive and the high-grade lesions, easy to distinguish from the fine cytoplasmic immunostain present in normal urothelium, reactive atypia and non-invasive low grade lesions. Although he has not found a direct correlation between p16 immunoexpression and tumor grade and stage, the authors concluded that this marker might be useful in preinvasive high-grade urothelial lesions. Exclusively cytoplasmic expression of p16 was demonstrated in tumor pathology of the mammary gland and the brain being considered a negative prognostic factor [17, 18].

Bartoletti R *et al.* [4], from a prospective study on p16 immunoexpression in urothelial carcinomas, has not found a statistical correlation between p16 and tumor grade or stage, however, suggesting a role of protein in the prediction of recurrence within the meaning of high risk expression relationship. Other studies have

suggested direct links between p16 immunoexpression and tumor stage, as is done in 2001 by Korkolopoulou P *et al.* [19], which revealed that the decrease of p16 expression in advanced stages of urothelial carcinoma, combined with p53 overexpression may establish prognosis of patients.

Protooncogene c-erbB2 (Her-2/neu) encodes a transmembrane glycoprotein similar to EGFR that has tyrosine kinase activity and stimulates cell growth [20]. It is a unique protein in the EGFR family, which present no specific ligand, signaling realizing by heterodimerisation with another member of same family [21].

In this study, c-erbB2 immunoexpression was identified in 67.8% of cases at the membrane level, the immunostain score being highest in case of moderate or poorly differentiated carcinomas with no any relation to tumor stage.

Data from the literature are controversial regarding the relationship between protein expression in urothelial level and histopathological prognostic parameters. Thus, Krüger S *et al.* reported in 2002, from a cohort of 203 patients, a correlation of protein expression with tumor grade and stage in 37% of investigated cases [22]. In another study made on 80 muscle invasive urothelial carcinoma, Jimenez RE *et al.* [23] have not found a direct correlation with stage or tumor grade but showed that in case of c-erbB2 overexpression tumors have an increased risk of metastasis, a conclusion confirmed by other studies [24]. This could suggest the therapeutic usefulness of protein expression in a manner similar to other locales, such as the mammary gland.

It confirmed that c-erbB2 amplification in urothelial carcinomas is less common than in other locales, which means that overexpression may have other less known biomolecular mechanisms, this being a possible obstacle in implementing an immunohistochemical protocol with therapeutic significance [25]. In most studies, c-erbB2 has not proven as independent prognostic marker although expression was higher in aggressive or advanced carcinoma [26–29].

Ki67 immunoexpression was identified in all cases and is considered positive for 78.5% of lesions analyzed, the rest having a proliferation index <10%.

Ki67 antigen is a non-histone protein with short life, expressed during the cell cycle phases G1, S, G2/M, being an indicator of tumor growth and aggressiveness [30]. Studies realized to now have determined that marker is an independent predictor for recurrence, progression and response to treatment for invasive urothelial carcinomas [31, 32]. In a study by Margulis V *et al.* on a sample of 226 patients with radical cystectomy and bilateral lymphadectomy, Ki67 expression was significantly associated with advanced stage, high tumor grade, lymph node invasion and lymphovascular metastases [33].

The study revealed a statistical correlation of Ki67/p16 and Ki67/c-erbB2 immunoexpression in the investigated urothelial carcinomas. Studies in the literature on the three biomarkers are numerous, but usually they were analyzed in the other panels of antibodies.

## Conclusions

The study indicated the involvement of p16 and c-erbB2 in proliferation and progression of bladder urothelial carcinomas. Although immunoexpression of the biomarkers used does not cover all urothelial tumor pathology, for a large part of it, p16 and c-erbB2 can provide important information on the prognosis of patients by correlating with tumor stage and grade. Relations with histopathological parameters are particularly important as it can be highlighted with a direct correlation of Ki67 with each of the two biomarkers.

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