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Ki67 and Bcl-2 immunoexpression in primitive urothelial bladder carcinoma

M. ENACHE[1,2)], CRISTIANA SIMIONESCU[2)], LUANA CORINA LASCU[3)]

[1)]*PhD candidate*
[2)]*Department of Pathology*
University of Medicine and Pharmacy of Craiova
[3)]*Department of Radiology and Medical Imaging, Emergency County Hospital, Craiova*

Abstract

Bladder cancer ranks among eight human malignant lesions, 90% being urothelial carcinomas. We evaluated the Ki67 and Bcl-2 immunoexpression and their correlations with clinicopathological parameters. The study included 45 primitive bladder urothelial carcinoma diagnosed in patients aged in VI and VII decades of life, predominantly in males. Histopathologically, the most numerous were moderately differentiated carcinomas (68.8%), most patients being classified in stage I of disease (48.8%). The analysis for Ki67 immunostain revealed positivity in 71.1% of cases, with higher values in moderate and poorly differentiated tumors in stage III or IV of disease. In contrast, immunoreactivity for Bcl-2 was present in 33.3% of well and moderately differentiated analyzed tumors and classified in stage I and II of disease. Tumor stage and grade is not correlated with Bcl-2 but there was a strong correlation with Ki67 proliferation index. Ki67 immunoexpression may be helpful to identify patients at high risk who may benefit by adjuvant therapies.

***Keywords*:** urothelial carcinoma, immunohistochemistry, Ki67, Bcl-2.

Introduction

Bladder cancer currently occupies eight place among worldwide human malignant lesions and represents 3.2% of these [1, 2], approximately 90% being urothelial carcinomas [3, 4].

Urothelial bladder carcinomas represent a unique model to investigate the cancer process, due to their biological behavior and endoscopic approach. Despite progresses made in surgical techniques, specific survival without disease at 5 years is 50–60% [5, 6]. In addition, clinical and pathological parameters have limited value in assessing tumor recurrence and progression, or survival of patients. Thus, tumor stage is the most important morphological prognostic factor in urothelial bladder carcinomas being determined by depth of invasion into the bladder wall [7]. Although tumor stage and grade are parameters that can cause therapeutic attitude, often cannot be predicted risk of progression because urothelial carcinomas are morphologically and genetically heterogeneous tumors, with various malignant cell lines and specific alterations [7].

The aim of the study is to evaluate the expression of Ki67 and Bcl-2 in primitive urothelial bladder carcinomas and also to identify statistically significant correlations with clinical and morphological analyzed parameters.

Materials and Methods

The study included 45 cases of primitive bladder urothelial carcinomas, diagnosed in the Pathology Laboratory of Emergency County Hospital of Craiova. The biological material was represented by cystectomy pieces, which were processed by common histopathological technique using 10% formalin fixation, paraffin embedding and Hematoxylin–Eosin stain. The histopathological diagnosis was done in conformity with criterions established in 2004 by *WHO* [8]. To assess the lymph node invasion and distant metastasis status was used the RMN investigation.

The immunohistochemical processing was made on serial sections using the LSAB+ System–HRP (DAKO, code K0690). The antibodies used, clone, dilution and antigenic recovery are shown in Table 1.

Table 1 – *Panel of used antibodies*

Antibody	Clone	Dilution	Antigen retrieval
Bcl-2	124	1:75	Citrate buffer, pH 6
Ki67	MIB1	1:100	Citrate buffer, pH 6

For quantification of immunological reactions, we used an adapted system of cases distribution according to the immunostain: score 3 – 50% labeled cells, score 2 – 10–50% labeled cells and score 1, negative – less than 10% labeled cells [9, 10]. To quantify Ki67 immunoexpression was calculated the proliferation index (IP), by dividing the number of positive cells to total cells in a 40× microscope field [11]. Negative external control staining was done by omitting primary antibodies. Statistical analysis of the results was performed in SPSS 10 software using the *chi*-square test for dependence assessment. The acquisition of the images was done with Nikon Eclipse E600 and software program Lucia 5.

Results

The study was performed on a group of 45 cases of primitive bladder urothelial carcinomas. Tumors were diagnosed in patients aged in VI and VII decades of life, predominantly in males (male/female rapport – 6.5/1) (Table 2).

Table 2 – *Clinicopathological parameters*

Clinicopathological parameters		No. of cases	%
Age [years]	51–60	21	46.6
	61–70	24	53.4
Gender	Male	39	86.6
	Female	6	13.4
Growth pattern	Papillary	43	95.5
	Non-papillary	2	4.5
Tumor grade	Well-differentiated	13	28.8
	Moderately differentiated	31	68.8
	Poorly differentiated	11	24.4
Tumor stage	I (T1N0M0)	22	48.8
	II (T2a,bN0M0)	15	33.3
	III (T3aN0M0)	5	11.1
	IV (T3N1/2M0)	3	6.6

Histopathologically, the examined tumors had predominantly a papillary growth pattern, present in 43 cases and only in two cases they developed a non-papillary one. In terms of the degree of differentiation, tumors were well-differentiated in 13 cases, moderately differentiated in 31 cases, and poorly differentiated in 11 cases (Figure 1, a and b; Table 2).

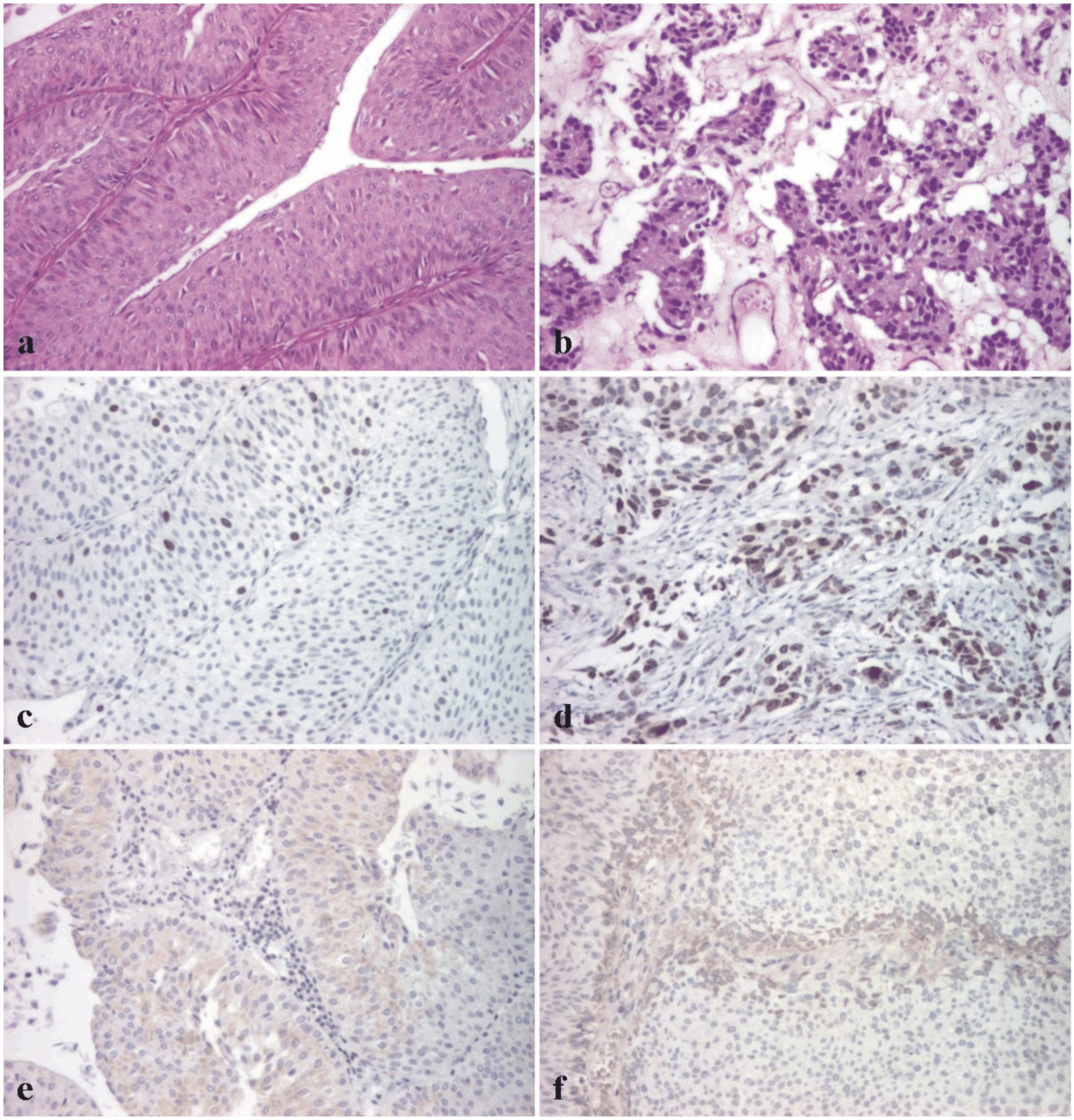

Figure 1 – ***(a) Well-differentiated urothelial carcinoma, HE stain, ×100; (b) Poorly differentiated urothelial carcinoma, HE stain, ×100; (c) Ki67 immunostain, well-differentiated urothelial carcinoma, ×100; (d) Ki67 immunostain, poorly differentiated urothelial carcinoma, ×100; (e) Bcl-2 immunostain, well-differentiated urothelial carcinoma, ×100; (f) Bcl-2 immunostain, moderately differentiated urothelial carcinoma, ×100.***

Clinical and morphological staging of tumors revealed that most cases were diagnosed in stage I of disease, 48.8% of cases respectively, followed by stage II, 33.3%, stage III with 11.1% and stage IV with 6.6% of cases (Table 2).

Immunohistochemical analysis pursued Ki67 and Bcl-2 expression and correlations with clinical and morphological parameters studied.

Ki67 immunoreaction was identified in all cases at nuclear level but was considered positive in 32 cases (71.1%), the remaining cases having a proliferation index <10% (Table 3).

Table 3 – ***Ki67 and Bcl-2 immunoexpression score according to tumor grade and stage***

Tumor grade/stage	Well-differentiated				Moderately differentiated				Poorly differentiated			
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Ki67 score	0	0	1	1	0	1	2	2	1	2	2	2
Bcl-2 score	3	3	0	0	2	2	1	1	0	0	0	0

Proliferation index values ranged between 5% and 62%, the highest values being recorded for moderately and poorly differentiated stage III or stage IV carcinomas (Figure 1d). In 36% and respectively 53% of well and moderately differentiated studied carcinomas, Ki67 proliferation index was <10% (Figure 1c).

Statistical analysis showed highly significant correlation between Ki67 proliferation index, tumor grade (p<0.001) and tumor stage (p<0.001).

Bcl-2 immunostain was present in 15 cases at cytoplasmic level, which represented 33.3% of the examined tumors. Bcl-2 positive urothelial carcinomas were well-differentiated (11 cases) or moderately differentiated (four cases) in stage I and stage II of disease.

In well-differentiated carcinomas immunoreaction has score 3, the average marked cells being 56% (Figure 1e). In moderately differentiated carcinomas immunoreaction has score 1 or 2, the average marked cells being 19% (Figure 1f).

Statistical analysis of cases showed a significant correlation of Bcl-2 with tumor grade (p<0.05) and it was no correlation with tumor stage and Ki67 proliferation index (p>0.05).

Discussion

Biomolecular pathogenic pathways that trigger the occurrence and development of bladder urothelial carcinoma are maintained by alternative mechanisms involving genes and proteins altered since the early stages of disease. For conventional urothelial carcinomas were evaluated many biological markers in order to better understand the molecular events leading to disease progression and to study the correlation with tumor stage and behavior, including Bcl-2 and MIB-1. Tumors with loss of Bcl-2 and p53 and Ki67 overexpression are considered to have poor prognosis but multivariate analysis indicated that none of them have independent prognostic value [12].

Uncontrolled proliferation of cells is considered one of the most important biological mechanisms associated with oncogenesis [13]. Ki67 is a non-histone nuclear protein with a short life, expressed by proliferating cells during the cell cycle phases G1, S, G2 / M. It can be immunohistochemically determined, nuclear expression allowing the appreciation of biological aggressiveness of a malignant tumor and is therefore an indicator of tumor growth and aggressiveness [14]. This marker is an independent predictor of patient outcomes in several malignancies [15, 16]. In urothelial tumors, growth potential of tumors is an important indicator of their aggressiveness [17, 18].

In this study, Ki67 proliferation index was positive in 32 cases (71.1%), with maximum in moderately and poorly differentiated carcinomas, being in stages T2–T4, and there was a highly significant correlation with tumor grade and stage (p<0.001).

The literature data indicates that Ki67 proliferation index has increased in high-grade carcinoma with or without invasion [19]. Studies carried until now have established that it is an independent predictor for recurrence, progression and response to treatment in invasive urothelial carcinoma [20, 21].

Several recent studies have established that Ki67 expression is an independent predictor of disease recurrence, progression, and the response to intravesical therapy for patients with noninvasive bladder cancer in the muscle layer [20–22]. However, few studies have examined the importance of Ki67 expression in patients with locally advanced or metastatic tumors. Popov Z *et al.* (1997), using a heterogeneous group of 114 patients treated with transurethral resection or radical cystectomy, has concluded that Ki67 expression was associated with the recurrence of disease [23]. In a cohort study of 75 patients treated with radical cystectomy, Suwa Y *et al.* (1997) found that Ki67 expression was an independent predictor of patient survival, but most patients in this series had locally advanced disease or nodal metastases [24]. Korkolopoulou P *et al.* (2000) and Krause FP *et al.* (1997) showed a significant correlation between histological grade and Ki67, noting that pTa and pT1 tumors had a Ki67 index lower than pT2–4 tumors [25, 26].

Another multi-institutional cohort study in patients treated with radical cystectomy for urothelial bladder carcinoma, validates strong predictive power of Ki67 by the data obtained, indicating that Ki67 has statistically significant predictive value, similar with other currently assessed parameters as tumor stage, lymphovascular invasion and lymph node status [27].

Programmed cell death or apoptosis, plays important role in tumor pathology, loss of apoptotic response of tumor cells represents one of the mechanisms involved in tumor progression and occurrence of relapses. Tumor growth rate is dependent by the rate of proliferation of neoplastic cells and the number of died cells, a process that occurs mainly by apoptosis [28]. Disruption of biomolecular mechanisms that control apoptosis in the bladder may result in survival of cells with genomic abnormalities, tumorigenesis and resistance to anticancer therapy [28].

Protein produced by the gene Bcl-2 is located in the internal mitochondrial membrane, with a role in stopping cell death and cell survival, without involving proliferation mechanisms [29]. In the normal urothelium, immunoreactivity for Bcl-2 occurs in the basal layer and undifferentiated cells that require protection against apoptosis [30].

In this study, we found Bcl-2 positivity in well or moderately differentiated carcinomas that have invaded up to the muscle, the maximum score being found in T1 well-differentiated carcinoma. Bcl-2 immunoexpression significantly correlated only with tumor grade ($p<0.05$).

Data from the literature regarding the significance of Bcl-2 immunoexpression in urothelial carcinomas are quite controversial. Some studies support the theory that tumor growth was due to the anti-apoptotic Bcl-2 is much slower than if the increase would be determined by proliferative factors, tumor cells becoming less responsive to such factors, with better prognosis for Bcl-2 positive tumors [12, 31]. Regardless of Bcl-2 expression, the authors agree that this pathway is significantly lower in the progression of lesions compared with other tumor locations.

In other studies Bcl-2 immunoexpression was correlated with less aggressive phenotype [30, 32]. It was reported in a such study that tumors with lamina propria invasion, poorly differentiated and aggressive, presented an increased Bcl-2 immunoexpression, also existing cases of well-differentiated superficial carcinomas Bcl-2 positive and cases of aggressive carcinomas with negative stain for this marker [33].

Conclusions

Stage and tumor grade of primitive bladder transitional carcinoma does not correlate with apoptosis, but there is a strong correlation with cell proliferation. Ki67 immunoexpression can improve the accuracy of prognosis, being helpful in identifying patients with high risk of disease progression, providing a valuable indicator for the clinical management of these patients, which may benefit by adjuvant therapies.

References

[1] Jemal A, Siegel R, Xu J, Ward E, *Cancer statistics, 2010*, CA Cancer J Clin, 2010, 60(5):277–300.

[2] Knowles MA, *Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese?* Carcinogenesis, 2006, 27(3):361–373.

[3] Pashos CL, Botteman MF, Laskin BL, Redaelli A, *Bladder cancer: epidemiology, diagnosis, and management*, Cancer Pract, 2002, 10(6):311–322.

[4] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM, *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008*, Int J Cancer, 2010, 127(12):2893–2917.

[5] Cookson MS, *The surgical management of muscle invasive bladder cancer: a contemporary review*, Semin Radiat Oncol, 2005, 15(1):10–18.

[6] Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, Skinner E, Bochner B, Thangathurai D, Mikhail M, Raghavan D, Skinner DG, *Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients*, J Clin Oncol, 2001, 19(3):666–675.

[7] Lopez-Beltran A, Cheng L, *Stage pT1 bladder carcinoma: diagnostic criteria, pitfalls and prognostic significance*, Pathology, 2003, 35(6):484–491.

[8] Lopez-Beltran A, Sauter G, Gasser T, Tumours of the urinary system. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IA (eds), *Pathology and genetics of tumours of the urinary system and male genital organs*, World Health Organization Classification of Tumours, IARC Press, Lyon, 2004, 89–154.

[9] Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM, Schalken JA, *Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors*, Cancer Res, 1993, 53(14):3241–3245.

[10] Desai S, Lim SD, Jimenez RE, Chun T, Keane TE, McKenney JK, Zavala-Pompa A, Cohen C, Young RH, Amin MB, *Relationship of cytokeratin 20 and CD44 protein expression with WHO/ISUP grade in pTa and pT1 papillary urothelial neoplasia*, Mod Pathol, 2000, 13(12):1315–1323.

[11] Compérat E, Camparo P, Haus R, Chartier-Kastler E, Bart S, Delcourt A, Houlgatte A, François R, Capron F, Vieillefond A, *Immunohistochemical expression of p63, p53 and MIB-1 in urinary bladder carcinoma. A tissue microarray study of 158 cases*, Virchows Arch, 2006, 448(3):319–324.

[12] Nakopoulou L, Vourlakou C, Zervas A, Tzonou A, Gakiopoulou H, Dimopoulos MA, *The prevalence of bcl-2, p53, and Ki-67 immunoreactivity in transitional cell bladder carcinomas and their clinicopathologic correlates*, Hum Pathol, 1998, 29(2):146–154.

[13] van Diest PJ, Brugal G, Baak JPA, *Proliferation markers in tumours: interpretation and clinical value*, J Clin Pathol, 1998, 51(10):716–724.

[14] Cattoretti G, Becker MH, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J, *Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections*, J Pathol, 1992, 168(4):357–363.

[15] Li R, Heydon K, Hammond ME, Grignon DJ, Roach M 3rd, Wolkov HB, Sandler HM, Shipley WU, Pollack A, *Ki-67 staining index predicts distant metastasis and survival in locally advanced prostate cancer treated with radiotherapy: an analysis of patients in radiation therapy oncology group protocol 86-10*, Clin Cancer Res, 2004, 10(12 Pt 1):4118–4124.

[16] Gimotty PA, Van Belle P, Elder DE, Murry T, Montone KT, Xu X, Hotz S, Raines S, Ming ME, Wahl P, Guerry D, *Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma*, J Clin Oncol, 2005, 23(31):8048–8056.

[17] Limas C, Bigler A, Bair R, Bernhart P, Reddy P, *Proliferative activity of urothelial neoplasms: comparison of BrdU incorporation, Ki67 expression, and nucleolar organiser regions*, J Clin Pathol, 1993, 46(2):159–165.

[18] Stepan A, Simionescu C, Mărgăritescu C, Ciurea R, *P16, c-erbB2 and Ki67 immunoexpression in urothelial carcinomas of the bladder*, Rom J Morphol Embryol, 2011, 52(2):653–658.

[19] Helpap B, Köllermann J, *Assessment of basal cell status and proliferative patterns in flat and papillary urothelial lesions: a contribution to the new WHO classification of urothelial tumors of the urinary bladder*, Hum Pathol, 2000, 31(6):745–750.

[20] Quintero A, Alvarez-Kindelan J, Luque RJ, Gonzalez-Campora R, Requena MJ, Montironi R, Lopez-Beltran A, *Ki-67 MIB1 labelling index and the prognosis of primary TaT1 urothelial cell carcinoma of the bladder*, J Clin Pathol, 2006, 59(1):83–88.

[21] Su JS, Arima K, Hasegawa M, Franco OE, Yanagawa M, Sugimura Y, Kawamura J, *Proliferative status is a risk index for recurrence in primary superficial (pTa/T1) low-grade urothelial bladder carcinoma*, Hinyokika Kiyo, 2003, 49(11): 649–658.

[22] Pich A, Chiusa L, Formiconi A, Galliano D, Bortolin P, Comino A, Navone R, *Proliferative activity is the most significant predictor of recurrence in noninvasive papillary urothelial neoplasms of low malignant potential and grade 1 papillary carcinomas of the bladder*, Cancer, 2002, 95(4): 784–790.

[23] Popov Z, Hoznek A, Colombel M, Bastuji-Garin S, Lefrere-Belda MA, Bellot J, Abboh CC, Mazerolles C, Chopin DK, *The prognostic value of p53 nuclear overexpression and MIB-1 as a proliferative marker in transitional cell carcinoma of the bladder*, Cancer, 1997, 80(8):1472–1481.

[24] Suwa Y, Takano Y, Iki M, Asakura T, Noguchi S, Masuda M, *Prognostic significance of Ki-67 expression in transitional cell bladder carcinoma after radical cystectomy*, Pathol Res Pract, 1997, 193(8):551–556.
[25] Korkolopoulou P, Christodoulou P, Kapralos P, Exarchakos M, Bisbiroula A, Hadjiyannakis M, Georgountzos C, Thomas-Tsagli E, *The role of p53, MDM2 and c-erb B-2 oncoproteins, epidermal growth factor receptor and proliferation markers in the prognosis of urinary bladder cancer*, Pathol Res Pract, 1997, 193(11–12):767–775.
[26] Krause FS, Feil G, Bichler KH, *Immunohistochemical examinations (Ki67, p53, nm23) and DNA cytophotometry in bladder cancer*, Anticancer Res, 2000, 20(6D):5023–5028.
[27] Margulis V, Lotan Y, Karakiewicz PI, Fradet Y, Ashfaq R, Capitanio U, Montorsi F, Bastian PJ, Nielsen ME, Müller SC, Rigaud J, Heukamp LC, Netto G, Lerner SP, Sagalowsky AI, Shariat SF, *Multi-institutional validation of the predictive value of Ki-67 labeling index in patients with urinary bladder cancer*, J Natl Cancer Inst, 2009, 101(2):114–119.
[28] McKnight JJ, Gray SB, O'Kane HF, Johnston SR, Williamson KE, *Apoptosis and chemotherapy for bladder cancer*, J Urol, 2005, 173(3):683–690.
[29] Leonardo C, Mattace Raso D, Albino G, Pozza M, Gallucci M, *The role of Bcl-2 and Bax genes for urinary bladder cancer relapse*, Eur Urol, 2003, 2(1 Suppl 2):45.
[30] Shiina H, Igawa M, Urakami S, Honda S, Shirakawa H, Ishibe T, *Immunohistochemical analysis of bcl-2 expression in transitional cell carcinoma of the bladder*, J Clin Pathol, 1996, 49(5):395–399.
[31] Ong F, Moonen LM, Gallee MP, ten Bosch C, Zerp SF, Hart AA, Bartelink H, Verheij M, *Prognostic factors in transitional cell cancer of the bladder: an emerging role for Bcl-2 and p53*, Radiother Oncol, 2001, 61(2):169–175.
[32] Şen Türk N, Aybek Z, Düzcan E, *Ki-67, p53, Bcl-2 and Bax expression in urothelial carcinomas of urinary bladder*, Türk Patoloji Dergisi, 2010, 26(1):25–30.
[33] Gonzalez-Campora R, Davalos-Casanova G, Beato-Moreno A, Garcia-Escudero A, Pareja Megia MJ, Montironi R, Lopez-Beltran A, *BCL-2, TP53 and BAX protein expression in superficial urothelial bladder carcinoma*, Cancer Lett, 2007, 250(2):292–299.

Corresponding author
Cristiana Simionescu, Professor, MD, PhD, Department of Pathology, University of Medicine and Pharmacy of Craiova, 66 1 May Avenue, 200628 Craiova, Romania; Phone/Fax +40251–599 228, e-mail: csimionescu2004@yahoo.com

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