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Proliferation activity in bladder tumors does not correlate with the pathological grading

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Abstract

Worldwide, bladder cancer is the seventh most frequent cancer in men and the 17th most frequent cancer in women, respectively. In men, this type of cancer is the second most frequent type of cancer localized in the genitourinary system, after prostate cancer. The incidence of bladder cancer is ever growing and the etiopathogenic factors of bladder cancer are numerous and still not fully understood. Smoking is the most common risk factor incriminated in the onset of urinary tract cancer, the incidence of bladder cancer being directly connected to the smoking duration and the tobacco amount intake. Regarding the histopathological types, more than 90% of bladder cancer is represented by transitional cell carcinomas. Histopathology assessment of bladder cancer is a constant challenge regarding the connection between tumor grading, depth of invasion, extension and clinical prognosis. We evaluated here a number of 32 confirmed bladder tumors and we aimed to find common patterns of expression for markers like cytokeratin 7 (CK7), CK20, vascular endothelial growth factor (VEGF), CD34, matrix metalloproteinases (MMPs) 2, 8 and 9, as well as for the Ki67 proliferation index. Our study showed that both CK7 and CK20 were present in different tumor areas and tumor gradings, MMP9 was more constantly expressed compared to the more variable expression of MMPs 2 and 8, vascular densities did not seem to increase in high-grade invasive tumors compared to low-grade tumors. Interestingly, while high Ki67 proliferating indexes were present especially in high-grade superficially tumors, compared to low-grade papillary tumors; this correlation was inversed for the advancing edges of the tumor. This common feature of invasive urothelial tumors will thus require further studies in order to elucidate the cellular signaling pathways by which these tumors increase their overall invasiveness.

Keywords: bladder cancer, progression, papillary tumors, squamous differentiation.

Introduction

Worldwide, bladder cancer is the seventh most frequent cancer in men and the 17th most frequent cancer in women, respectively [1, 2]. In men, this type of cancer is the second most frequent type of cancer localized in the genital urinary tract, after prostate cancer.

In 2012, all over the world, there were diagnosed about 330 000 new cases of bladder cancer and about 123 000 patients deceased because of this disease [3].

In 2012, in Europe, the estimated incidence of bladder cancer was 151 300, of which 118 400 cases were diagnosed in men and 32 900 in women [4]. In Europe, as well, bladder cancer in men is the second most common malignant condition of the urinary tract, after prostate cancer [4, 5]. In the USA, bladder cancer is quite a common malignity, being the fifth most common form of cancer, every year being diagnosed approximately 74 000–75 000 new cases [6].

The incidence of bladder cancer is ever growing. In 2016, in the USA there were diagnosed about 76 960 new cases and there were recorded about 16 390 deaths caused by this disease [7].

The etiopathogenic factors of bladder cancer are numerous and still not fully understood. Smoking is the most common risk factor incriminated in the onset of urinary tract cancer, the incidence of bladder cancer being directly connected to the smoking duration and the tobacco amount intake [8]. Other risk factors are represented by chronic infections of the urinary tract, arsenic exposure and professional exposure to carcinogen agents from the rubber and fossil fuel industry, a diet poor in vitamins and oligoelements, genetic factors, etc. [9–13].

Regarding the histopathological tumor types, more than 90% of bladder cancers are represented by transitional cell carcinomas, 5% by squamous cell carcinomas and 2% by adenocarcinomas, with the existence of multiple differentiation forms in each pure histological subtype [14].

Urothelial carcinomas are classified in two large categories: non-invasive bladder cancers that limits to the urothelial cell layers (Ta or Tis stages) or they just penetrate the lamina propria (T1 stage); and the invasive bladder tumors that involve the muscularis propria of the

organ. Approximately 75–80% of all bladder carcinomas recently diagnosed are non-invasive in the bladder muscles and 20–25% are invasive ones [15].

In our study, we aim at highlighting the histological and immunohistochemical (IHC) characteristics of bladder tumors and to correlate their microscopic aspects with the tumor proliferation index.

Materials and Methods

Our study included 32 bladder tumor fragments harvested during a transurethral endoscopic resection of the tumor formations present in the bladder, together with those from the 32 patients diagnosed clinically, paraclinically and cytoscopically with bladder tumors. The resected tumor fragments were fixed in 10% formalin, pH 7.4, for 48 hours, after which they were processed by the common histopathological technique of paraffin inclusion. Histological sections were obtained on a Microm HM350 rotary microtome, equipped with a section transfer system on water bath (STS, Microm, Germany). The histopathological study was performed on Hematoxylin–Eosin (HE) stained samples. For the IHC study, the histological sections were collected on poly-L-lysine covered slides. The section immunostaining followed the classical protocol: section deparaffination and hydration, antigen retrieval by boiling the sections in a sodium citrate solution (pH 6) for 21 minutes in a microwave oven, blocking of endogenous peroxidase by immersion of sections in 3% water peroxide, for 30 minutes, at room temperature, and blocking the non-specific sites by immersion of sections in 2% skimmed milk for 30 minutes. The sections were then incubated with primary antibodies, for 18 hours (overnight), in a refrigerator, at 4^0C. The next day, there was applied the biotinylated secondary antibody for 30 minutes, at room temperature, after which there was applied Streptavidin–Horseradish peroxidase (HRP) for 30 minutes. The signal was detected by using 3.3'-Diaminobenzidine (DAB) (Dako). There followed the contrasting with Mayer's Hematoxylin, alcohol dehydration, xylene clarification and fixing the blades by using a DPX environment (Fluka).

In our study, we used the following antibodies: anti-cytokeratin 20 (CK20) (clone Ks20.8, 1/25 dilution, Dako), anti-CK7 (clone OV-TL 12/30, 1/50 dilution, Dako), anti-Ki67 (clone MIB-1, 1/50 dilution, Dako), anti-CD34 (clone QBEnd 10, 1/50 dilution, Dako), anti-vascular endothelial growth factor (VEGF) (clone VG1, 1/100 dilution, Dako), anti-matrix metalloproteinase 2 (MMP2) (clone 8B4, 1/50 dilution, Novus Biologicals), anti-MMP8 (clone EP1252Y, 1/50 dilution, Abcam), anti-MMP9 (polyclonal, 1/150 dilution, Dako).

Imaging was done on a Nikon Eclipse 55i microscope equipped with a Nikon DS-5Mc cooled charge-coupled device (CCD) camera and the Image-Pro Plus AMS software package (MediaCybernetics, Silver Spring, MD, USA). For Ki67 index, tumor areas were randomly photographed under a 40× objective and then positive nuclei were expressed as percentage from the total epithelial nuclei. Statistical comparison was performed utilizing a Student's *t*-test. Data are expressed as average ± standard deviation.

Results

In most of the cases (80%), histopathology identified papillary tumors, with increased cellularity of the covering urothelium, and variable nuclear atypia (Figure 1, A–C). In 75% of the cases, the diagnostic was of non-invasive urothelial carcinomas, either as low-grade, or as high-grade. In low-grade tumors, one could identify branched papillary stalks and variations in nuclear shape and size, polarity and chromatin appearance. Nucleoli were not present all the time, and mitoses were infrequent. There was no invasion into the adjacent lamina propria or beyond that (Figure 1, A–C). For high-grade tumors, the papillary architecture was less regulate, with frequently fused and branched papillae (Figure 1, D–E). Nuclear variation was more evident than in low-grade tumors, with higher pleomorphism, variations in nuclear polarity, large nucleoli and more frequent mitoses.

Invasive urothelial carcinomas, besides being papillary or non-papillary, revealed minimal to blunt invasion in lamina propria and beyond (Figure 1F). Infiltrative tumors without any papillary component showed irregular and angulated tumor islands dissecting into the lamina propria and submucosae (Figure 1, G and H), sometimes retaining a clear-cut urothelial phenotype, while in some cases there was a complete anaplasia with hemorrhagic areas and tumor necrosis (Figure 1I). In these poorly differentiated tumors (Figure 1J), sometimes we could identify regions of squamous differentiation (Figure 1K), or mucinous differentiation (Figure 1L). In 30% of these cases, we identified invasion of the muscularis mucosae smooth muscle cell fibers (Figure 1M), most of these fragments being resected from posterior tumors located in the bladder trigone. Nerve invasion was identified in 12% of the cases (Figure 1N), while intravascular emboli were a relative rare event (Figure 1O).

CK20 expression was limited especially at the upper layers of the epithelium for papillary tumors, but on occasion it span through the whole thickness of the epithelium (Figure 2, A and B). In less differentiated areas, the staining tended to become diffuse and less intense (Figure 2C). CK20 was present in 71.88% of all investigated tumors, most frequently the negative tumors being high-grade carcinomas. CK7 was usually expressed in all the layers of the tumoral epithelium (Figure 2D), sometimes being more intense in the basal layer but retaining a diffuse-like pattern for the remaining layers (Figure 2, E and F). CK7 was expressed in all tumors investigated.

On the superficial tumor areas, Ki67 expression was increased in high-grade papillary carcinomas (83.14± 15.21%) compared to low-grade carcinomas (41.23± 12.32%), $p<0.001$ with Student's *t*-test (Figure 2, G–I; Figure 3). However, when assessing the deeper parts of the tumor epithelium, proliferative cells tended to be more homogenous in the center of the tumor islands

for both low-grade (58.45±18.42%) and high-grade invasive tumors (64.31±14.57%) (Figure 2, J–L), with no statistical differences between Ki67 index values (p>0.05, Student's t-test) (Figure 3). Although for high-grade tumors, the index tended to be higher in the surface areas compared to deep invasive areas, there was no statistical significance between these values (p>0.05, Student's t-test).

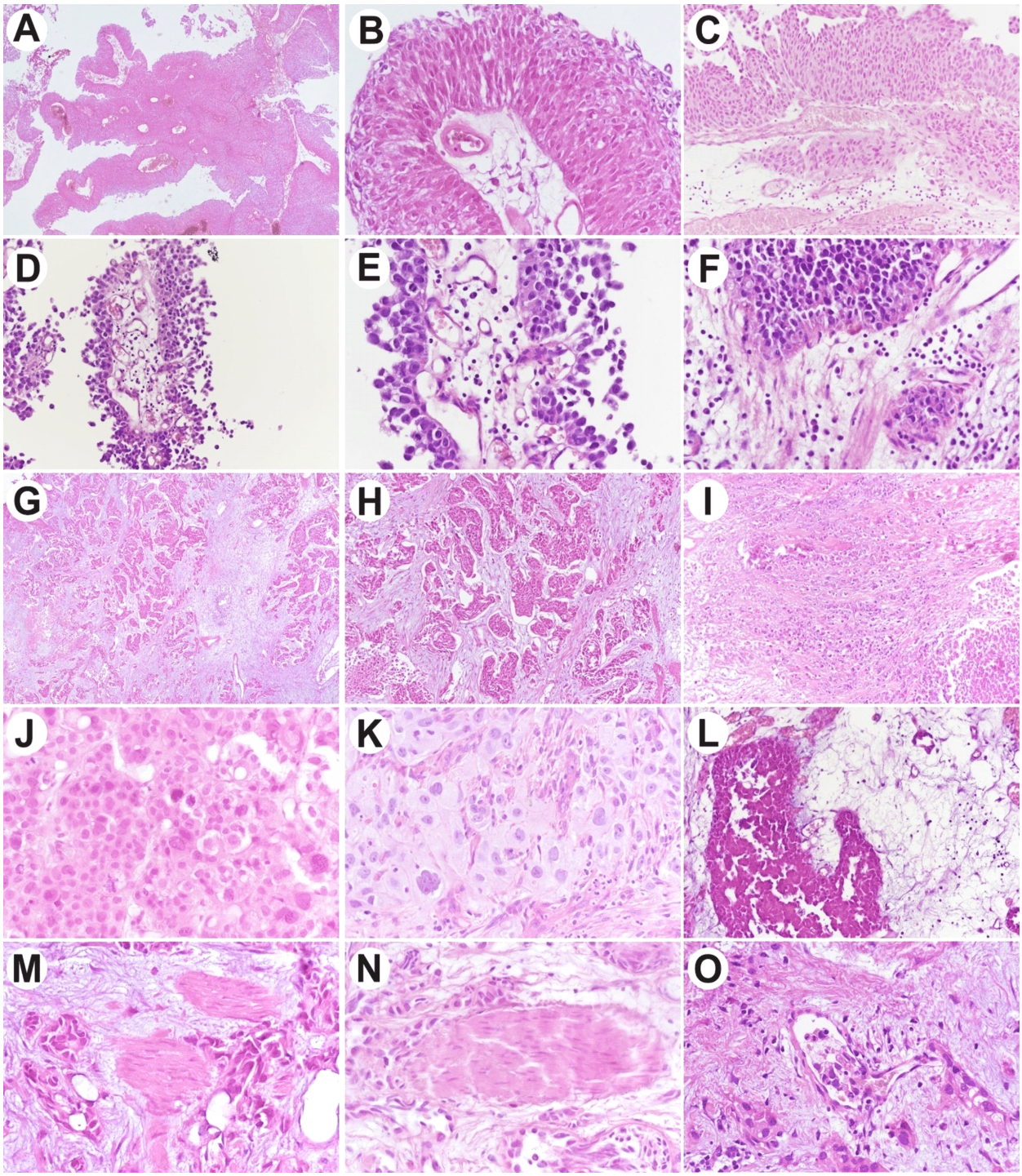

Figure 1 – ***Histopathology of non-invasive and invasive urothelial tumors: (A–C) Non-invasive low-grade papillary carcinoma; (D and E) High-grade papillary carcinomas with invasion into the lamina propria (F); (G and H) Deep infiltrative tumor islands, sometimes associated with necrosis and hemorrhagic areas (I); High-grade tumors reveal accentuated cellular pleomorphism (J and K), and areas of variety differentiation, exemplified here by squamous (K) and mucinous differentiation (L); Disease extension features included invasion of the muscularis mucosae (for tumors located in the bladder trigone) (M), nerve invasion (N), or vascular tumor emboli (O). HE staining: (A) ×40; (B, C, G, H, I, and L) ×100; (D–F, J–O) ×200.***

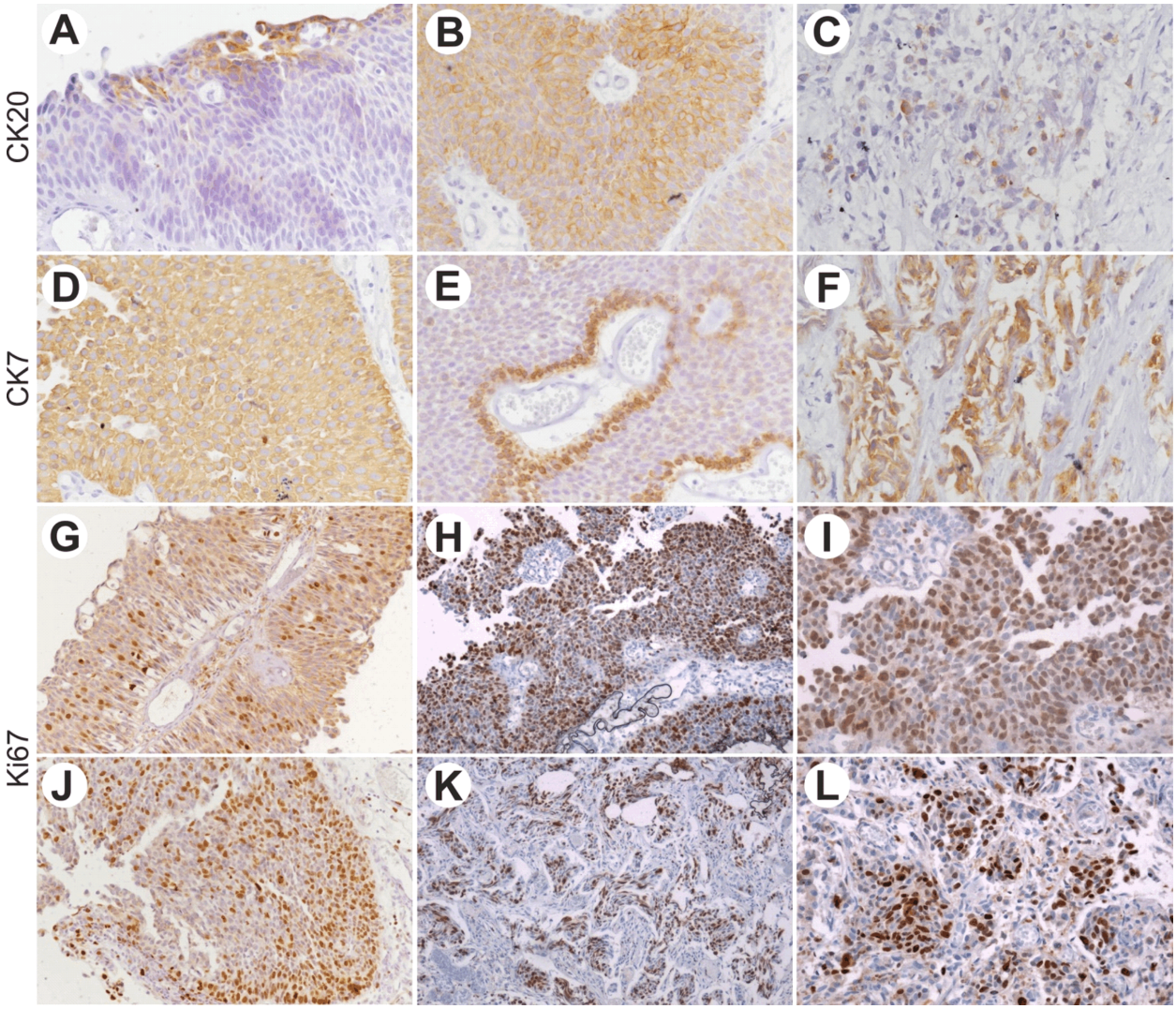

Figure 2 – ***Immunophenotype of urothelial tumors (part I): (A) CK20 is expressed especially in the upper epithelial layers of superficial tumors, but on occasion, it is present variable in the depth of the tumors (B and C); CK7 is mostly expressed through the epithelium (D), in the basal layers (E), or variable in the invading areas (F); Ki67 seems to have a lower index for low-grade superficial tumors (G), compared to high-grade superficial tumors (H and I), but with overall lower values for invasive tumors (J–L). Anti-CK20 antibody immunostaining: (A and B) ×100; (C) ×200. Anti-CK7 antibody immunostaining: (D and E) ×100; (F) ×200. Anti-Ki67 antibody immunostaining: (G, H, J, and K) ×100; (I and L) ×200.***

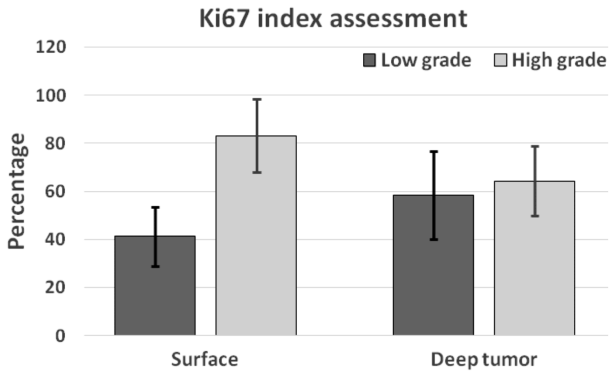

Figure 3 – ***Ki67 index shows significantly higher values in high grade superficial tumors compared to invasive areas, Student's t-test, p<0.001. Error bars represent standard deviation.***

Vascular density was not higher for invasive tumors (Figure 4, A and B), but was increased for inflammatory areas with granulation tissue (Figure 4C). That is, the presence of more tumor stromal component did not necessarily lead to an associated increase of vascular density. VEGF was variably expressed in low-grade tumors, but was negative or with a very low expression for high-grade tumors (Figure 4, D–F). MMPs 2 and 8 showed a granular expression in the tumor cells, for both low-grade and high-grade tumors, and were present also to a lesser extent in the stromal cells of the tumor stroma (Figure 4, G and H). For MMP9, there was the same strong expression in the tumor cells for all tumors, but the stromal cells expressed it much lesser compared to MMPs 2 and 8 (Figure 4I). Inflammatory infiltrate was less abundant in low-grade tumors compared to high-grade tumors. For high-grade tumors, T-cells tended to be more numerous compared to B-cells (Figure 4, J–L).

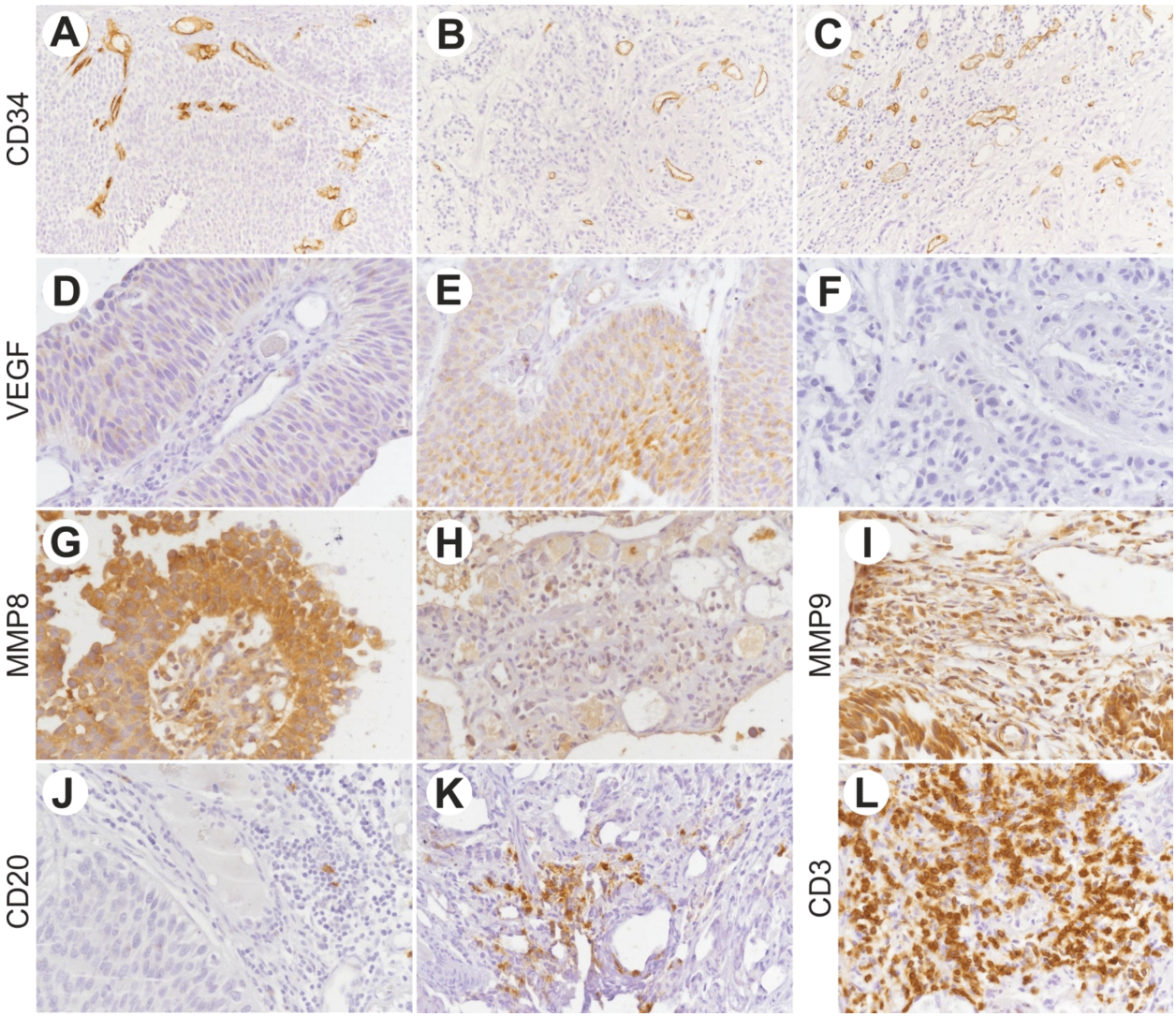

Figure 4 – ***Immunophenotype of urothelial tumors (part II): Stromal and vascular density seems comparable in non-invasive (A) versus invasive tumors (B and C); VEGF was variable in low-grade tumors (D and E) and mostly negative in high-grade tumors (F); MMP8 was variable positive (G and H), with less variable MMP9 expression (I); Inflammatory infiltrate was variable, but mostly consisted of T-cells rather than B-lymphocytes (J–L). Anti-CD34 antibody immunostaining: (A–C) ×100. Anti-VEGF antibody immunostaining: (D–F) ×200. Anti-MMP8 antibody immunostaining: (G and H) ×200. Anti-MMP9 antibody immunostaining: (I) ×200. Anti-CD20 antibody immunostaining: (J and K) ×200. Anti-CD3 antibody immunostaining: (L) ×200.***

Discussion

Our study showed that bladder neoplasm is a heterogeneous condition, regarding the pathological and IHC aspects, suggesting that, in the disease etiopathogeny, there are incriminated, in various proportions, numerous external or internal factors. Of the risk factors incriminated in the etiopathogeny of bladder tumors, clinical studies showed that smoking is the greatest risk factor, being involved in about 50% of bladder cancers [3, 8, 16]. The fact that smoking is the main etiological factor also explains the 3–4 times higher incidence of bladder cancer in men (the most numerous smokers), in comparison to women [17, 18]. Besides smoking, there were also identified other risk factors, such as professional exposure to certain chemical substances (polycyclic flavored hydrocarbons, chlorocarbons) [19], pelvic irradiations for malignant conditions of the organs in this anatomical region, certain medicines used in the treatment of a pre-existent cancer or for treating diabetes [20–22], excessive alcohol intake, renal lithiasis, infections (bacterial, parasitic, fungi and viral) and bladder inflammations [23–27], genetic and epigenetic changes, etc. We believe that all these etiological factors have a chronic, cumulative action, leading in time to the onset of dysplasia, metaplasia and ultimately to bladder neoplasia. Therefore, bladder cancer most often appears over the age of 65 years old [18, 28].

The pathological study performed by us showed that most cases of bladder tumors presented a papillary aspect. Thus, of the 32 cases of bladder tumors, 25 (80%) were papillary ones. Our data are similar to those found in other studies showing that papillary forms are the most frequent ones [29, 30].

Tumor papillary lesions of bladder take extremely varied clinical, macroscopic and pathological forms, from benign lesions (papillomas), to high malignant lesions and

with a threatening prognosis [31]. Most of the primary bladder tumors (90–95%) are represented by urothelial cell carcinomas [5, 32]. The *World Health Organization* (WHO) published a classification for urothelial papillary tumors, where bladder papillomas can also take a borderline form called papillary neoplasms with a low malignant potential, as the differentiation between the urothelial papilloma in papillary carcinomas, especially in non-invasive ones, is sometimes difficult to be made only based on the pathological characteristics [32, 33].

In our study, for differentiating the urothelial carcinomas from papillomas, we took into consideration cellular polymorphism, changes of the nucleus/cytoplasm ratio, presence of nucleoli, presence of cell mitoses, etc. Nevertheless, we considered, along with other authors [34] that a high contribution to establishing a differential diagnosis of these tumors may be provided by immunohistochemistry techniques.

CK20 was identified in our study in 23 cases. It appeared as more intense in the superficial urothelial layer. In the poorly differentiated tumors, immunostaining became less intense and more diffuse, having the tendency to stain the deeper layers, as well. Still, the immunoreaction to CK7 appeared more intense in the basal layer and more diffuse in the intermediary and superficial layers. Cytokeratins are a family of polypeptides that belong to the structure of intermediary filaments from the cellular cytoskeleton [34]. In the tumor processes, they present abnormal IHC expressions. Thus, CK20 is present in some urothelial tumor cells, being a marker that predicts the malignant potential of urothelial tumors or a marker for urothelial cell dysplasia [35]. It seems that the CK20 expression is as intense as the carcinoma is more differentiated and it is more reduced and diffuse in poorly differentiated carcinomas [36]. Other studies support the idea that the presence of the CK20 reaction in the whole tumoral urothelium thickness or a complete lack of an IHC reaction for this marker could be a sign of high aggressiveness of the tumoral cells and a sign of the disease recurrence [37].

Investigating the cellular proliferation marker Ki67 in our study showed that this was high in highly non-invasive papillary carcinomas, still, opposite to our expectations, it was more reduced in highly invasive tumors. Ki67 is an IHC marker expressed in the nuclei of the cells under division, more precisely in stages G1, S, G2 and M of the cellular cycle. Due to this reason, Ki67 is a diagnosis and prognosis instrument frequently used in characterizing malignant tumors [38–42]. Still, some studies showed that immunomarker Ki67 cannot mark all the proliferative cellular fraction, especially the cells in stage G1 of the cellular cycle. Therefore, the detection by IHC staining of this marker may vary from one tumor to another [43, 44]. It is possible that this explanation could be reliable in our study, as well. However, most studies showed that the IHC marker Ki67 is extremely useful for evaluating the cellular proliferation, being at the same time a reliable prognosis indicator for bladder tumors [45–47].

In our study, the vascular changes in the tumor stroma, the presence of the inflammatory infiltrate and the MMPs reaction may be a direct consequence of the immune system reaction to the presence of tumor cells and/or of the inflammatory reaction induced by urinary infections. Various clinical and experimental studies showed that in bacterial infection, especially with *Escherichia coli*, this may play both a major and a synergetic part during the carcinogenesis of the bladder [48, 49]. Chronic inflammation of the bladder caused by microorganisms may also interfere with the pathogeny of bladder cancer, by stimulating the increase of cancer cells, the invasion and metastasizing, by recruiting inflammatory cells and signaling molecules at the carcinogenesis spot [50, 51]. Other times, bladder infections may represent a complication of bladder cancer.

Conclusions

Overall, our study illustrated the variable immunoexpression patterns of different markers in urothelial cancer. Importantly, we showed here that Ki67 exhibit the same variability, and even it tends to correlate inversely with the degree of tumor invasiveness, this observation being thus of clinico-pathological importance for the overall assessment of urothelial tumor aggressivity.

Conflict of interests

The authors declare that they have no conflict of interests.

References

[1] Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, Kassouf W, Kiemeney LA, La Vecchia C, Shariat S, Lotan Y. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol, 2013, 63(2):234–241.

[2] Klaile Y, Schlack K, Boegemann M, Steinestel J, Schrader AJ, Krabbe LM. Variant histology in bladder cancer: how it should change the management in non-muscle invasive and muscle invasive disease? Transl Androl Urol, 2016, 5(5):692–701.

[3] Andreassen BK, Aagnes B, Gislefoss R, Andreassen M, Wahlqvist R. Incidence and survival of urothelial carcinoma of the urinary bladder in Norway 1981–2014. BMC Cancer, 2016, 16(1):799.

[4] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, 2015, 136(5):E359–E386.

[5] Benhamou S, Bonastre J, Groussard K, Radvanyi F, Allory Y, Lebret T. A prospective multicenter study on bladder cancer: the COBLAnCE cohort. BMC Cancer, 2016, 16(1):837.

[6] Dadhania V, Czerniak B, Guo CC. Adenocarcinoma of the urinary bladder. Am J Clin Exp Urol, 2015, 3(2):51–63.

[7] Li HT, Duymich CE, Weisenberger DJ, Liang G. Genetic and epigenetic alterations in bladder cancer. Int Neurourol J, 2016, 20(Suppl 2):S84–S94.

[8] Freedman ND, Silverman DT, Hollenbeck AR, Schatzkin A, Abnet CC. Association between smoking and risk of bladder cancer among men and women. JAMA, 2011, 306(7):737–745.

[9] Brandau S, Böhle A. Bladder cancer. I. Molecular and genetic basis of carcinogenesis. Eur Urol, 2001, 39(5):491–497.

[10] Feng Z, Hu W, Rom WN, Beland FA, Tang MS. 4-Aminobiphenyl is a major etiological agent of human bladder cancer: evidence from its DNA binding spectrum in human p53 gene. Carcinogenesis, 2002, 23(10):1721–1727.

[11] Kiemeney LA, Sulem P, Besenbacher S, Vermeulen SH, Sigurdsson A, Thorleifsson G, Gudbjartsson DF, Stacey SN, Gudmundsson J, Zanon C, Kostic J, Masson G, Bjarnason H, Palsson ST, Skarphedinsson OB, Gudjonsson SA, Witjes JA, Grotenhuis AJ, Verhaegh GW, Bishop DT, Sak SC,

Choudhury A, Elliott F, Barrett JH, Hurst CD, de Verdier PJ, Ryk C, Rudnai P, Gurzau E, Koppova K, Vineis P, Polidoro S, Guarrera S, Sacerdote C, Campagna M, Placidi D, Arici C, Zeegers MP, Kellen E, Gutierrez BS, Sanz-Velez JI, Sanchez-Zalabardo M, Valdivia G, Garcia-Prats MD, Hengstler JG, Blaszkewicz M, Dietrich H, Ophoff RA, van den Berg LH, Alexiusdottir K, Kristjansson K, Geirsson G, Nikulasson S, Petursdottir V, Kong A, Thorgeirsson T, Mungan NA, Lindblom A, van Es MA, Porru S, Buntinx F, Golka K, Mayordomo JI, Kumar R, Matullo G, Steineck G, Kiltie AE, Aben KK, Jonsson E, Thorsteinsdottir U, Knowles MA, Rafnar T, Stefansson K. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. Nat Genet, 2010, 42(5):415–419.

[12] Figueroa JD, Ye Y, Siddiq A, Garcia-Closas M, Chatterjee N, Prokunina-Olsson L, Cortessis VK, Kooperberg C, Cussenot O, Benhamou S, Prescott J, Porru S, Dinney CP, Malats N, Baris D, Purdue M, Jacobs EJ, Albanes D, Wang Z, Deng X, Chung CC, Tang W, Bas Bueno-de-Mesquita H, Trichopoulos D, Ljungberg B, Clavel-Chapelon F, Weiderpass E, Krogh V, Dorronsoro M, Travis R, Tjønneland A, Brenan P, Chang-Claude J, Riboli E, Conti D, Gago-Dominguez M, Stern MC, Pike MC, Van Den Berg D, Yuan JM, Hohensee C, Rodabough R, Cancel-Tassin G, Roupret M, Comperat E, Chen C, De Vivo I, Giovannucci E, Hunter DJ, Kraft P, Lindstrom S, Carta A, Pavanello S, Arici C, Mastrangelo G, Kamat AM, Lerner SP, Barton Grossman H, Lin J, Gu J, Pu X, Hutchinson A, Burdette L, Wheeler W, Kogevinas M, Tardón A, Serra C, Carrato A, García-Closas R, Lloreta J, Schwenn M, Karagas MR, Johnson A, Schned A, Armenti KR, Hosain GM, Andriole G Jr, Grubb R 3rd, Black A, Ryan Diver W, Gapstur SM, Weinstein SJ, Virtamo J, Haiman CA, Landi MT, Caporaso N, Fraumeni JF Jr, Vineis P, Wu X, Silverman DT, Chanock S, Rothman N. Genome-wide association study identifies multiple loci associated with bladder cancer risk. Hum Mol Genet, 2014, 23(5):1387–1398.

[13] Golabek T, Bukowczan J, Sobczynski R, Leszczyszyn J, Chlosta PL. The role of micronutrients in the risk of urinary tract cancer. Arch Med Sci, 2016, 12(2):436–447.

[14] Olivier Bosset P, Neuzillet Y, Paoletti X, Molinie V, Botto H, Lebret T. Long-term follow-up of TaG1 non-muscle-invasive bladder cancer. Urol Oncol, 2015, 33(1):20.e1–20.e7.

[15] Babjuk M, Böhle A, Burger M, Capoun O, Cohen D, Compérat EM, Hernández V, Kaasinen E, Palou J, Rouprêt M, van Rhijn BW, Shariat SF, Soukup V, Sylvester RJ, Zigeuner R. EAU Guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2016. Eur Urol, 2017, 71(3):447–461.

[16] Gottlieb J, Higley C, Sosnowski R, Bjurlin MA. Smoking-related genitourinary cancers: a global call to action in smoking cessation. Rev Urol, 2016, 18(4):194–204.

[17] Scosyrev E, Noyes K, Feng C, Messing E. Sex and racial differences in bladder cancer presentation and mortality in the US. Cancer, 2009, 115(1):68–74.

[18] Shakhssalim N, Hosseini SY, Basiri A, Eshrati B, Mazaheri M, Soleimanirahbar A. Prominent bladder cancer risk factors in Iran. Asian Pac J Cancer Prev, 2010, 11(3):601–606.

[19] Brown T, Slack R, Rushton L; British Occupational Cancer Burden Study Group. Occupational cancer in Britain. Urinary tract cancers: bladder and kidney. Br J Cancer, 2012, 107(Suppl 1): S76–S84.

[20] Pedersen-Bjergaard J, Ersbøll J, Hansen VL, Sørensen BL, Christoffersen K, Hou-Jensen K, Nissen NI, Knudsen JB, Hansen MM. Carcinoma of the urinary bladder after treatment with cyclophosphamide for non-Hodgkin's lymphoma. N Engl J Med, 1988, 318(16):1028–1032.

[21] Mamtani R, Haynes K, Bilker WB, Vaughn DJ, Strom BL, Glanz K, Lewis JD. Association between longer therapy with thiazolidinediones and risk of bladder cancer: a cohort study. J Natl Cancer Inst, 2012, 104(18):1411–1421.

[22] Zeng FC, Cen S, Tang ZY, Kang XL. Elevated matrix metalloproteinase 9 expression may contribute to the pathogenesis of bladder cancer. Oncol Lett, 2016, 11(3):2213–2222.

[23] Parkin DM. The global burden of urinary bladder cancer. Scand J Urol Nephrol Suppl, 2008, (218):12–20.

[24] Pasin E, Josephson DY, Mitra AP, Cote RJ, Stein JP. Superficial bladder cancer: an update on etiology, molecular development, classification, and natural history. Rev Urol, 2008, 10(1):31–43.

[25] Stone L. Bladder cancer: urinary tract infection increases risk. Nat Rev Urol, 2015, 12(1):4.

[26] Richards KA, Ham S, Cohn JA, Steinberg GD. Urinary tract infection-like symptom is associated with worse bladder cancer outcomes in the Medicare population: implications for sex disparities. Int J Urol, 2016, 23(1):42–47.

[27] Sui X, Lei L, Chen L, Xie T, Li X. Inflammatory microenvironment in the initiation and progression of bladder cancer. Oncotarget, 2017, 8(54):93279–93294.

[28] Malats N, Real FX. Epidemiology of bladder cancer. Hematol Oncol Clin North Am, 2015, 29(2):177–189, *vii*.

[29] Mehta N, Rathore RS, Pillai BS, Sam MP, Moorthy K. Intrinsic tumour factors affecting recurrence in non muscle invasive bladder cancer: a hospital based study from India. Asian Pac J Cancer Prev, 2015, 16(7):2675–2677.

[30] Heney NM. Natural history of superficial bladder cancer. Prognostic features and long-term disease course. Urol Clin North Am, 1992, 19(3):429–433.

[31] Poyet C, Hermanns T, Zhong Q, Drescher E, Eberli D, Burger M, Hofstaedter F, Hartmann A, Stöhr R, Zwarthoff EC, Sulser T, Wild PJ. Positive fibroblast growth factor receptor 3 immunoreactivity is associated with low-grade non-invasive urothelial bladder cancer. Oncol Lett, 2015, 10(5):2753–2760.

[32] Alrashidy M, Atef A, Baky TA. Immunohistochemical differentiation between urothelial papillomas and papillary neoplasms of low malignant potential of the urinary bladder. Asian Pac J Cancer Prev, 2016, 17(4):1769–1772.

[33] 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 (WHO) Classification of Tumours, International Agency for Research on Cancer (IARC) Press, Lyon, France, 2004, 90–110.

[34] Poiană C, Carşote M, Ardeleanu C, Terzea D, Avramescu ET, Neamţu MC, Miulescu RD. The value of the immunohistochemistry in a case of gastric neuroendocrine tumor and thyroid metastasis. Rom J Morphol Embryol, 2011, 52(1): 187–192.

[35] Alsheikh A, Mohamedali Z, Jones E, Masterson J, Gilks CB. Comparison of the WHO/ISUP classification and cytokeratin 20 expression in predicting the behavior of low-grade papillary urothelial tumors. World Health Organization/International Society of Urologic Pathology. Mod Pathol, 2001, 14(4):267–272.

[36] Mumtaz S, Hashmi AA, Hasan SH, Edhi MM, Khan M. Diagnostic utility of p53 and CK20 immunohistochemical expression grading urothelial malignancies. Int Arch Med, 2014, 7:36.

[37] Harnden P, Mahmood N, Southgate J. Expression of cytokeratin 20 redefines urothelial papillomas of the bladder. Lancet, 1999, 353(9157):974–977.

[38] 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.

[39] Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol, 2000, 182(3):311–322.

[40] Lopez-Beltran A, Luque RJ, Alvarez-Kindelan J, Quintero A, Merlo F, Carrasco JC, Requena MJ, Montironi R. Prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S modulators (p53, p21Waf1, p27kip1, Cyclin D1, and Cyclin D3) and proliferation index (Ki67-MIB1). Eur Urol, 2004, 45(5):606–612.

[41] Falato C, Lorent J, Tani E, Karlsson E, Wright PK, Bergh J, Foukakis T. Ki67 measured in metastatic tissue and prognosis in patients with advanced breast cancer. Breast Cancer Res Treat, 2014, 147(2):407–414.

[42] Barbu CG, Florin A, Neamţu MC, Avramescu ET, Terzea D, Miron A, Dănciulescu Miulescu R, Poiană C, Fica S. Papillary thyroid carcinoma with anaplastic dedifferentiation in the lymph node metastasis – a rare form of presentation even for a tall cell variant. Rom J Morphol Embryol, 2015, 56(2):527–531.

[43] Hashimoto K, Araki K, Osaki M, Nakamura H, Tomita K, Shimizu E, Ito H. MCM2 and Ki-67 expression in human lung adenocarcinoma: prognostic implications. Pathobiology, 2004, 71(4):193–200.

[44] Burger M, Denzinger S, Hartmann A, Wieland WF, Stoehr R, Obermann EC. Mcm2 predicts recurrence hazard in stage

Ta/T1 bladder cancer more accurately than CK20, Ki67 and histological grade. Br J Cancer, 2007, 96(11):1711–1715.
[45] Mulder AH, Van Hootegem JC, Sylvester R, ten Kate FJ, Kurth KH, Ooms EC, Van der Kwast TH. Prognostic factors in bladder carcinoma: histologic parameters and expression of a cell cycle-related nuclear antigen (Ki-67). J Pathol, 1992, 166(1):37–43.
[46] Rudolph P, Peters J, Lorenz D, Schmidt D, Parwaresch R. Correlation between mitotic and Ki-67 labeling indices in paraffin-embedded carcinoma specimens. Hum Pathol, 1998, 29(11):1216–1222.
[47] Pfister C, Lacombe L, Vezina MC, Moore L, Larue H, Têtu B, Meyer F, Fradet Y. Prognostic value of the proliferative index determined by Ki-67 immunostaining in superficial bladder tumors. Hum Pathol, 1999, 30(11):1350–1355.
[48] El-Mosalamy H, Salman TM, Ashmawey AM, Osama N. Role of chronic *E. coli* infection in the process of bladder cancer – an experimental study. Infect Agent Cancer, 2012, 7(1):19.
[49] Vermeulen SH, Hanum N, Grotenhuis AJ, Castaño-Vinyals G, van der Heijden AG, Aben KK, Mysorekar IU, Kiemeney LA. Recurrent urinary tract infection and risk of bladder cancer in the Nijmegen bladder cancer study. Br J Cancer, 2015, 112(3):594–600.
[50] Nesi G, Nobili S, Cai T, Caini S, Santi R. Chronic inflammation in urothelial bladder cancer. Virchows Arch, 2015, 467(6): 623–633.
[51] Anderson-Otunu O, Akhtar S. Chronic infections of the urinary tract and bladder cancer risk: a systematic review. Asian Pac J Cancer Prev, 2016, 17(8):3805–3807.

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