Analysis of bladder cancer subtypes in neurogenic bladder tumors

Quentin Manach, MD,[1] Olivier Cussenot, MD,[2] Morgan Rouprêt, MD,[1] Xavier Gamé, MD,[3] Emmanuel Chartier-Kastler, MD,[1] Christine Reus, MD,[1] Philippe Camparo, MD,[4] Eva Compérat, MD,[2] Véronique Phé, MD[1]

[1]Sorbonne Université, GRC n°5, ONCOTYPE-URO, AP-HP, Hôpital Pitié-Salpêtrière, F-75013, Paris, France

[2] Sorbonne Université, GRC n°5, ONCOTYPE-URO, AP-HP, Hôpital Tenon, F-75020, Paris, France

[3]Department of Urology, Rangueil Academic Hospital, Toulouse, France

[4]Pathology Center, Amiens, France

MANACH Q, CUSSENOT O, ROUPRET M, GAME X, CHARTIER-KASTLER E, REUS C, CAMPARO P, COMPERAT E, PHE V. Analysis of bladder cancer subtypes in neurogenic bladder tumors. *Can J Urol* 2018;25(1):9161-9167.

Introduction: *To establish if the validated tumor biomarkers of luminal and basal bladder cancers in non neuro-urological patients are applicable to a neuro-urological population.*

Materials and methods: *We retrieved bladder cancer samples from neuro-urological patients (n = 20) and non-neurological controls (n = 40). The expression of GATA3 and CK5/6 was analyzed using immunohistochemistry of microarray tissue sections. We also assessed the correlation between previous biomarker expression, gender, age, tumor stage (non-muscle-invasive bladder cancer (NMIBC)/muscle-invasive bladder cancer (MIBC)), squamous-cell differentiation and basal/luminal subtypes using Pearson's correlation coefficient (r).*

Results: *Mean age at diagnosis of bladder cancer in neuro-urological patients was 53.2 years (min 41-max 73). MIBC was found in 13 neuro-urological patients (65%). The luminal subtype was identified in 7 samples (35%, all urothelial differentiation). The basal subtype was found in 13 samples (65%): 12 squamous-cell and 1 sarcomatoid differentiation. GATA3 and CK5/6 were expressed in 6 (30%) neuro-urological patients. A significant positive correlation was found between GATA3 expression and the luminal subtype (p = 0.00001, r = 0.5676). This was not the case with the neuro-urological status (r = -0.307). A poor correlation was found between CK5/6 expression and the neuro-urological status (r = 0.471 and -0.471), squamous-cell differentiation (r = 0.092), tumor stage NMIBC/MIBC (r = -0.118 and 0.118) and basal/luminal subtypes (r = -0.157 and 0.194).*

Conclusion: *In summary, the expression of GATA3 and CK5/6 could not differentiate the different subtypes of bladder cancer in neuro-urological patients. This implies that their specific histopathological signature is distinct from non neuro-urological patients. Additional pathways may be involved to explain their urothelial carcinogenesis mechanism.*

Key Words: basal, neurogenic bladder, urothelial carcinoma, bladder cancer, luminal

Introduction

Current therapeutic management for bladder cancer is made based on histopathological characteristics. Despite some inter-observer discrepancies, histopathology is the most accurate, cost-effective and fastest method for treatment decisions such as intravesical BCG instillation or neoadjuvant chemotherapy. Bladder cancer can be divided into two molecular subtypes, luminal and basal, with distinct clinical behaviors and sensitivities to chemotherapy.[1,2] These subtypes differ from the usual morphology and express different protein markers.[1,2] According to research data, basal subtype tumors (squamous cell carcinoma-like) have a more aggressive behavior at presentation and are sensitive to neoadjuvant chemotherapies. In contrast, luminal subtype tumors are more sensitive

Accepted for publication November 2017

Address correspondence to Professor Morgan Rouprêt, Department of Urology, Pitié-Salpêtrière Academic Hospital, 47-83 Boulevard de l'Hôpital, 75651 Paris Cedex 13, France

to cisplatin-based chemotherapy.[1,2] Recently some authors have offered a therapeutic approach based only on the molecular subtype, suggesting that protein expression on paraffin embedded tissue might suffice for treatment decision making.[3] Furthermore, GATA3 is reported as a luminal subtype marker whereas CK5/6 is referred to as a basal subtype marker. The immunohistochemical expressions only, should be able to identify the molecular subtypes of bladder cancer with an accuracy of over 90%,[3] suggesting that a simple two-marker immunohistochemical classifier could be used for prognostic and therapeutic stratifications.

Although the incidence of bladder cancer is equivalent to the normal population,[4] neuro-urological patients (i.e with a neurogenic bladder) are prone to develop specifically squamous, sarcomatoid or muscle-invasive types of bladder cancer. These patients exhibit particular inflammatory processes involving T regulator lymphocyte (Tregs) pathways with Foxp3.[5] Even though the risk of developing bladder cancer in this specific group of patients is well known, there is no recommendation regarding their management, especially in MIBC with squamous differentiation. Therefore, there is a need to develop a more precise, biology-based approach to the classification of bladder cancer to guide clinical management in this specific population.

Consequently, we wondered whether these tumors could also be divided into the same molecular groups as non-neurogenic bladder cancer, knowing that probably some other pathways play a role in the carcinogenesis. Our aim was to investigate whether the validated tumor biomarkers of luminal and basal bladder cancer in non neuro-urological patients could be applied to bladder cancer in patients with neurogenic bladder.

Materials and methods

Tissue samples and tissue microarray (TMA) construction

Non-muscle-invasive and muscle-invasive bladder cancer tissue samples, issued from transurethral bladder resections and cystectomies from neuro-urological patients, were retrieved and compared to control samples, after obtaining written informed consent from the patients. For every bladder cancer tissue sample from a neuro-urological patient (n = 20), two bladder cancer tissue samples from a non-neuro-urological patient (n = 40), of the same grade, stage and age, were selected. The collection and use of bladder-tissue samples were performed after approval from the local ethics committee and were in accordance with all the relevant French laws, regulations, and codes of practice.

The original slides were reviewed independently by two senior uropathologists and were classified according to the WHO 2004 classification. The tumor stage (pT) was established according to the TNM 2009 Classification of Malignant Tumors.[6] A TMA was constructed using archived formalin-fixed paraffin-embedded bladder samples. Slides containing tumor and non-tumor tissues were selected and labeled with colored ink. For each case, three cores of the tumor (0.6 cm in diameter) were transferred from the selected area to the recipient block. Serial 3-µm sections of the TMA block were generated and stained with hematoxylin and eosin to verify that the cores adequately represented the diagnostic areas.

Immunohistochemistry of the TMA sections

Immunohistochemistry for GATA3 and CK5/6 was performed on the 3-µm TMA sections, as described previously.[7,8] Antigen retrieval was performed by incubating the deparaffinized and rehydrated 3-µm tissue sections in buffer containing 10 mM Tris and 1 mM ethylene-diamine tetra-acetic acid (EDTA) (pH 9.0) in a water-bath at 97°C for 30 minutes. Then, endogenous enzyme activity was blocked for 8 minutes using the enzyme blocker from the kit.

Immunohistochemistry was performed using the modified streptavidin-biotin-peroxidase method and diaminobenzidine as the chromogen (brown staining developed for 2 minutes). The antibodies used included mouse polyclonal anti CK5/6 (Ventana Medical Systems, AZ, USA, 1/100), and anti GATA 3 (Biocare, Concord, CM405B, 1/100). Finally, the sections were lightly counterstained with Mayer's hematoxylin (Labonord, Templemars, France) and were mounted using aqueous medium (Glycergel, Dako). The negative-control tissue was incubated with the absence of antibody. Three high-power fields (x400) were selected from the tumor and non-tumor tissues. Expressions of GATA3 and CK5/6 were analyzed in the tumor and non-tumor tissues. GATA3 and CK5/6 staining was considered positive in case of nuclear (for GATA3) membranous staining (for CK5/6), and if their expression was ≥ 10%.[9]

Statistical analysis

The following data were collected prospectively: gender, neurological disease, voiding mode, history of recurrent urinary-tract infections, presence of bladder stones, smoking, type of specific treatment (cystectomy, chemotherapy, radiation therapy), death and cause of death. The follow up time was defined as the time from initial diagnosis until the date of death, or the

time of the last follow up. Data were stratified for the statistical analysis as follows: age < or ≥ 65 years, gender, tumor stage (either non-muscle invasive bladder cancer –NMIBC- or muscle-invasive bladder cancer - MIBC), tumor subtype (luminal or basal), differentiation (either squamous or non-squamous), GATA3 expression (positive/negative) and CK5/6 expression (positive/negative). Analysis was carried out with XLSTAT software. The different correlations were tested using the Pearson's correlation coefficient (r). A p value < 0.05 was considered significant.

Results

Overall, mean age of the 20 neuro-urological patients with bladder cancer (15 men and 5 women) was 53.2 years (41-73). Ten (50%) had spinal-cord injury, 2 (10%) had multiple sclerosis, 1 (5%) had a brain stroke and 7 (35%) had spina bifida. Eight (40%) patients were performing intermittent self-catheterization and an indwelling catheter was required in two (10%) patients. Symptomatic recurrent urinary tract infections, bladder stones and smoking were reported in 8 (40%), 1 (5%) and 11 (55%) patients, respectively. Median duration of the neurological disease before diagnosis of bladder cancer was 41 years (IQR 24-46). Table 1 shows the histopathological characteristics of the studied population.

Non-invasive muscle bladder cancers were found in 7 patients (35%): 1 pTa low grade (5%), 3 pTa high grade (15%), 3 pT1 (15%). Muscle-invasive bladder cancers were found in 13 patients (65%): 1 pT2 (5%), 7 pT3 (35%) and 5 pT4 (25%). Squamous-cell differentiation was observed in 12 samples (65%). Basal subtype bladder cancer was found in 13 patients (65%) and luminal subtype in 7 (35%). Cystectomy was performed in 16/20 patients (80%), among which two had received prior BCG therapy. Among neuro-urological patients with a basal subtype bladder cancer, n = 2 received adjuvant chemotherapy, n = 1 received adjuvant radiochemotherapy and n = 1 adjuvant radiotherapy.

Median follow up time was 22 months (IQR 3.5-59.7). At the last follow up, 15/20 patients had died: of these 13 died from evolution of the oncological disease. GATA3 and CK/5/6 were expressed in 6 (30%) neuro-urological patients, Figures 1, 2a and 2b.

In non neuro-urological patients, GATA3 was expressed in 25 (62.5%) samples. CK5/6 was never expressed in non neuro-urological patients. A significant positive correlation was found between GATA3 expression and the luminal subtype (p = 0.00001, r = 0.5676). By contrast the expression of GATA3 was not

TABLE 1. **Histopathological characteristics of bladder cancers in neuro-urological and non neuro-urological patients**

	Overall population n = 60	**Neuro-urological patients n = 20**	**Non-neuro-urological patients n = 40**
Mean age, years (min-max)	64.6 (41-91)	53.2 (41-73)	68.9 (43-91)
Gender			
Male, n (%)	46 (76.7%)	15 (75%)	31 (77.5%)
Female, n (%)	14 (23.3%)	5 (25%)	9 (22.5%)
Tumor stage			
NMIBC, n (%)	21 (35%)	7 (35%)	14 (35%)
MIBC, n (%)	39 (65%)	13 (65%)	26 (65%)
Luminal-subtype bladder cancer	37 (61.7%)	7 (35%)	30 (75%)
Urothelial differentiation, n (%)	32 (53.3%)	7 (35%)	25 (62.5%)
Micropapillary differentiation, n (%)	2 (3.3%)	0	2 (5%)
Other, n (%)	3 (5%)	0	3 (7.5%)
Basal-subtype bladder cancer	23 (38.3%)	13 (65%)	10 (25%)
Squamous-cell differentiation, n (%)	22 (36.7%)	12 (60%)	10 (25%)
Sarcomatoid differentiation, n (%)	1 (1.6%)	1 (5%)	0
Positive GATA3 expression, n (%)	31 (51.7%)	6 (30%)	25 (62.5%)
Negative GATA3 expression, n (%)	29 (48.3%)	14 (70%)	15 (37.5%)
Positive CK5/6 expression, n (%)	6 (10%)	6 (30%)	0
Negative CK5/6 expression, n (%)	54 (90%)	14 (70%)	40 (100%)

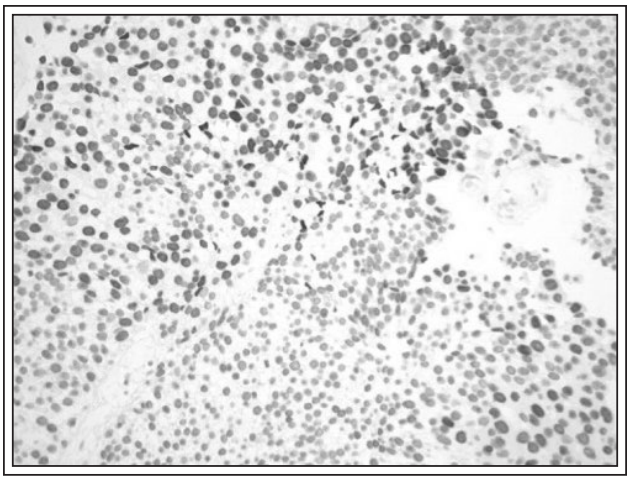

Figure 1. Strong nuclear GATA3 staining in a classical urothelial carcinoma of the bladder from a neuro-urological patient (x20).

significantly correlated to the neuro-urological status, the tumor stage or the squamous-cell differentiation.

A poor correlation was found between CK5/6 expression and neuro-urological status (r = 0.471 and -0.471), squamous-cell differentiation (r = 0.092), tumor stage NMIBC/MIBC (r = -0.118 and 0.118) and basal/luminal subtypes (r = -0.157 and 0.194).

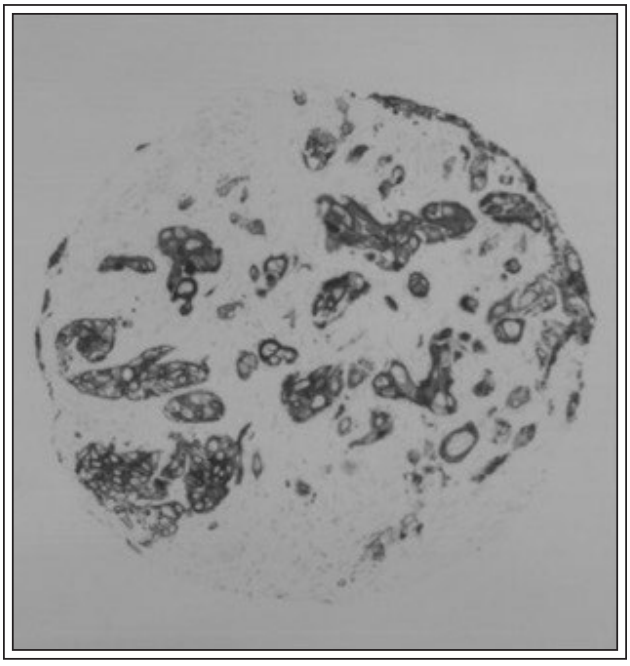

Figure 2a. Strong membranous staining of CK5/6 in a squamous urothelial carcinoma of the bladder from a neuro-urological patient (x10).

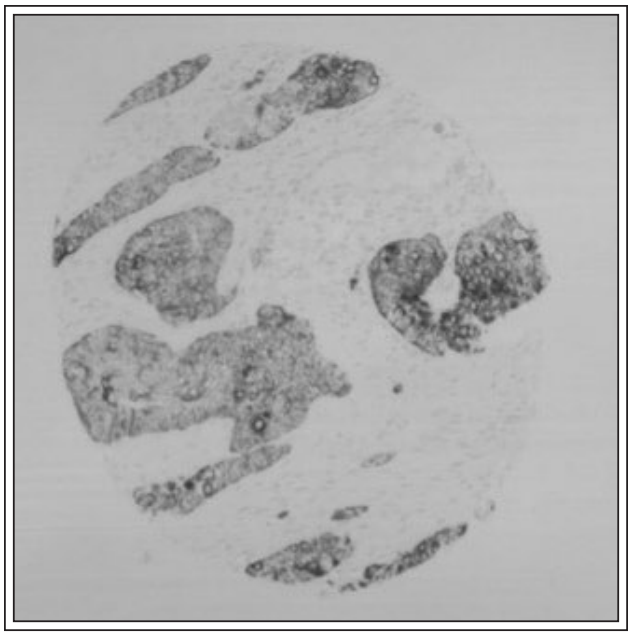

Figure 2b. Strong membranous staining of CK5/6 in a classical urothelial carcinoma of the bladder from a neuro-urological patient (x10).

A significant positive correlation was found between the luminal subtype and the non-muscle invasive stage (p = 0.000094, r = 0.4757).

A significant positive correlation was also established between the basal subtype and age < 65 years (p = 0.044738, r = 0.2565). A negative significant correlation was observed between male gender and the muscle invasive stage (p = 0.009087, r = -0.3065), See Table 2.

Discussion

To date, very few studies have reported on carcinogenesis within the neurogenic bladder,[5] particularly with regards to identifying the immunohistochemical diagnostic biomarkers.

The present study investigated whether the GATA3 and CK5/6 biomarkers could help differentiating between basal and luminal subtypes of bladder cancer in patients with a neurogenic bladder. Our preliminary results suggest that bladder cancers in neuro-urological patients have a specific histopathological signature different from non neuro-urological patients. GATA3 and CK5/6 were expressed in 6 (30%) neuro-urological patients.

A significant positive correlation was found between GATA3 expression and luminal subtype independently of the neuro-urological status, the tumor stage or the squamous-cell differentiation. These results suggest

that luminal subtype tumors may share the same carcinogenesis in neuro-urological and in non neuro-urological populations. The great majority of them are of conventional urothelial differentiation.

Although expressed in 30% of neuro-urological patients and never in non neuro-urological patients, a poor correlation was found between CK5/6 expression and neuro-urological status, squamous-cell differentiation, tumor stage NMIBC/MIBC, and basal/luminal subtypes.

Squamous features are generally a characteristic of basal bladder cancers and CK5/6 is a biomarker of squamous-cell differentiation in non neuro-urological patients.[10] The squamous histological features are considered distinct from conventional urothelial bladder cancers.

TABLE 2. **Pearson's correlation coefficients between each characteristic (neuro-urological status, gender, age, NMIBC/MIBC, CK5/6 and GATA3 expression**

Variables	Neuro-urological	Non neuro-urological	Male	Female	Age < 65y	Age ≥ 65y	NMIBC	MIBC	Squamous-cell differentiation	CK5/6 positive expression	GATA3 positive expression	Luminal-subtype BC	Basal-subtype BC
Neuro-urological	**1**												
Non neuro-urological	-1.000	**1**											
Male	-0.028	0.028	**1**										
Female	0.028	-0.028	-1.000	**1**									
Age < 65 y	0.236	-0.236	-0.042	0.042	**1**								
Age ≥ 65 y	-0.236	0.236	0.042	-0.042	-1.000	**1**							
NMIBC	0.025	-0.025	0.307	-0.307	-0.118	0.118	**1**						
MIBC	-0.025	0.025	-0.307 *p = 0.00909*	0.307	0.118	-0.118	-1.000	**1**					
Squamous-cell differentiation	0.342	-0.342	-0.234	0.234	0.226	-0.226	-0.391	0.391	**1**				
CK5/6 positive expression	0.471	-0.471 *p = 0 .00014*	-0.079	0.079	0.089	-0.089	-0.118	0.118	0.092	**1**			
GATA3 positive expression	-0.307	0.307	0.176	-0.176	-0.169	0.169	0.401	-0.401	-0.441	-0.233	**1**		
Luminal-subtype BC	-0.309	0.309	0.233	-0.233	-0.211	0.211	0.476 *p = 0.00009*	-0.476	-0.870	-0.157	0.568 *p = 0.00001*	**1**	
Basal-subtype BC	0.388	-0.388 *p = 0.00271*	-0.213	0.213	0.257 *p = 0.004474*	-0.257	-0.412	0.412	0.965	0.194	-0.472	-0.902	**1**

y = years; NMIBC = non-muscle-invasive bladder cancer; MIBC = muscle-invasive bladder cancer; BC = bladder cancer

The development of bladder cancers in neuro-urological patients predominantly occurs after a long period of progression of a neuro-urologic disease (frequently 15-20 years). Various risk factors, such as smoking, indwelling or supra-pubic catheterization, chronic urinary-tract infections and bladder stones, have been identified.[11,12] Moreover, bladder cancers in neuro-urological patients often display squamous-cell differentiation.[13]

In our study, among neuro-urological patients, 60% of bladder cancers displayed squamous-cell differentiation and 50% of them were MIBC. These results are in accordance with previous studies that have demonstrated that squamous-cell carcinoma typically presents at a more advanced local stage.[14] Inflammation caused by chronic irritation appears to play a role in the development of this type of differentiation.[12] More precisely a specific type of chronic inflammation, distinct from the classical inflammatory pathways, seems to be involved in the development of bladder cancers in neuro-urological patients.[5]

In a previous study, an elevated expression of Foxp3, a specific transcription factor of T-regulator lymphocytes (Tregs) appeared to be a characteristic in neuro-urological patients presenting with aggressive bladder cancers and squamous-cell differentiation.[5] Tregs play an important role in the maintenance of immunological self-tolerance by suppressing immune responses against autoimmune diseases and cancer.[15] Future studies need to determine the exact role of inflammation on the initiation and/or promotion of urothelial carcinogenesis and should focus in greater detail on the risk factors that increase bladder inflammation, examine genetic susceptibilities to inflammatory pathways and, when possible, include markers of inflammation before a cancer is diagnosed.

The immunohistochemical expression of only two markers, GATA3 and CK5/6 has been reported to be sufficient to identify the molecular subtypes of bladder cancers with over 90% accuracy,[3] suggesting that a simple two-marker immunohistochemical classifier can be used for prognostic and therapeutic stratification. This is not sufficient for neuro-urological patients. The link between squamous features and basal subtype is not proven in neuro-urological patients. To date no molecular or research data exist on this topic.

In the field of bladder cancers, current challenges are to improve the ability to accurately stage patients and to predict which patients will respond to which chemotherapy protocols. The ultimate goal is to allow personalized treatment plans. Various investigators are actively engaged in elucidating the biology of bladder cancer to assist/aid in the development of biomarkers that can predict disease aggressiveness and response to specific therapies. Treatment selection depends heavily on clinico-pathological features, but current staging systems are woefully inaccurate.[16] We tried to better understand the features of bladder cancers in neuro-urological patients. In our cohort, heterogeneity of the adjuvant treatment offered to patients depended on TNM stage. The search for a diagnostic biomarker for bladder cancer is a main goal given the importance of the problem in neuro-urological patients, the diagnosis at an advanced stage of the disease and the limited diagnostic usefulness of cystoscopy and cytology.[17]

We acknowledge a limitation to our study. The small number of patients limits the power of the study. However, the collection of such tissue samples from neuro-urological patients is unique and difficult to obtain, although performed in an expert center. In addition, this was a preliminary and exploratory study therefore we could not rely on the literature to compare our results. Since the information on the development of bladder cancers in neuro-urological patients is scarce, we thought that the approach to check for the involvement of biomarkers that have already been shown to play a role in non neuro-urological cohorts was valid. Based on the uniqueness of bladder cancer in neuro-urological patients, larger prospective studies should be conducted over the coming years.

Conclusion

The study of the expression of GATA3 and CK5/6 could not differentiate the different subtypes of bladder cancers in neuro-urological patients. Bladder cancers in neuro-urological patients have a specific histopathological signature different from non neuro-urological patients. Additional pathways might be involved in urothelial carcinogenesis of the neurogenic bladder. Further research is required to assess specific biomarkers. The ultimate goal is to help decision-making based on the histopathological signature. □

References

1. Sjödahl G, Lauss M, Lövgren K et al. A molecular taxonomy for urothelial carcinoma. *Clin Cancer Res* 2012;18(12):3377-3386.
2. Choi W, Porten S, Kim S et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. *Cancer Cell* 2014; 25(2):152-165.

3. Dadhania V, Zhang M, Zhang L et al. Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use. *EBioMedicine* 2016;12:105-117.
4. Parra J, Drouin S, Comperat E et al. Bladder cancer in neurological patients: analysis of a single-centre series. *Prog Urol* 2007;17(7):1333-1336.
5. Phé V, Rouprêt M, Cussenot O, Chartier-Kastler E, Gamé X, Compérat E. Forkhead box protein P3 (Foxp3) expression serves as an early chronic inflammation marker of squamous cell differentiation and aggressive pathology of urothelial carcinomas in neurological patients. *BJU Int* 2015;115 (Suppl 6):28-32.
6. Eble J, Sauter G, Epstein J, Sesterhenn I, Eds. World Health Organization Classification of Tumors. Pathology and genetics of tumors of the Urinary System and Male Genital Organs. IARC Press, Lyon, 2004. n.d.
7. Compérat E, Egevad L, Camparo P et al. Clinical significance of intratumoral CD8+ regulatory T cells in prostate carcinoma. *Anal Quant Cytol Histol* 2010;32(1):39-44.
8. Valdman A, Jaraj SJ, Compérat E et al. Distribution of Foxp3, CD4- and CD8-positive lymphocytic cells in benign and malignant prostate tissue. *APMIS* 2010;118(5):360-365.
9. Kobayashi K, Matsumoto H, Matsuyama H et al. Clinical significance of CD44 variant 9 expression as a prognostic indicator in bladder cancer. *Oncol Rep* 2016;36(5):2852-2860.
10. Gaisa NT, Braunschweig T, Reimer N et al. Different immunohistochemical and ultrastructural phenotypes of squamous differentiation in bladder cancer. *Virchows Arch* 2011;458(3):301-312.
11. Welk B, McIntyre A, Teasell R, Potter P, Loh E. Bladder cancer in individuals with spinal cord injuries. *Spinal Cord* 2013;51(7): 516-521.
12. West DA, Cummings JM, Longo WE, Virgo KS, Johnson FE, Parra RO. Role of chronic catheterization in the development of bladder cancer in patients with spinal cord injury. *Urology* 1999;53(2):292-297.
13. Pannek J. Transitional cell carcinoma in patients with spinal cord injury: a high risk malignancy? *Urology* 2002;59(2):240-244.
14. Rausch S, Hofmann R, von Knobloch R. Nonbilharzial squamous cell carcinoma and transitional cell carcinoma with squamous differentiation of the lower and upper urinary tract. *Urol Ann* 2012;4(1):14-18.
15. Ziegler SF. FOXP3: Not just for regulatory T cells anymore. *Eur J Immunol* 2007;37(1):21-23.
16. Svatek RS, Shariat SF, Novara G et al. Discrepancy between clinical and pathological stage: external validation of the impact on prognosis in an international radical cystectomy cohort. *BJU Int* 2011;107(6):898-904.
17. Sammer U, Walter M, Knüpfer SC, Mehnert U, Bode-Lesniewska B, Kessler TM. Do we need surveillance urethro-cystoscopy in patients with neurogenic lower urinary tract dysfunction? *PloS One* 2015;10(10):e0140970.