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RESEARCH AND CONTRIBUTIONS ON MARKERS INVOLVED IN URINARY BLADDER CANCER DIAGNOSIS, STAGING, PROGNOSTIC EVALUATION AND THERAPY

ABSTRACT

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INTRODUCTION

Malignant bladder tumors play an important role in the pathology of the genitourinary system. A slight increase in the number of cases of urinary bladder cancer (UBC) has been observed globally over the past 10 years [1]. The latest statistical report published in 2020 by the International Agency for Research on Cancer (IARC) estimated that in Romania, UBC was the 5th most common malignant tumor [1].

The most important classification of bladder cancer, from the perspective of therapy and disease progression, is the one that divides these tumors into non-muscle-invasive bladder carcinomas (NMIBC) and muscle-invasive bladder carcinomas (MIBC). Although there is a good survival rate for patients with NMIBC, more than half (60%) will experience recurrences within the next 5 years, and 20% will progress to MIBC. On the other hand, MIBC, defined as a tumor that infiltrates the muscularis propria (MP), represents an aggressive form of cancer with an increased capacity for invasion and metastasis [2]. Thus, identifying tumor invasion in the MP is an essential criterion for the establishment of the treatment, progression, and prognosis of patients with bladder cancer and a widely debated subject in the pathology community.

Advancements in molecular biology and interpreting the carcinogenesis of bladder neoplasms have opened the era of personalized medicine for patients with bladder carcinomas (BC). Various research groups involved in studying the molecular characteristics of MIBC have proposed different molecular classifications without reaching a consensus to date. To be applied in clinical practice, these molecular classifications must be validated, standardized, and universally accepted.

Remarkable progress in the therapy of BC has been made in recent decades through the introduction of immunotherapy (immune checkpoint inhibitors - ICI). The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved pembrolizumab, atezolizumab, nivolumab, avelumab, and durvalumab for second-line treatment of adult patients with urothelial carcinoma (UC) [3].

AIM AND OBJECTIVES OF THE STUDY

The main goals were to contribute to the management of patients with UC, to identify histopathological factors with potential value for the prognosis and therapy of this neoplasm. To achieve these goals, our main objectives were:

- outlining a clinical-morphological profile of patients with bladder cancer diagnosed in a reference center in the western region of Romania;
- evaluating potential IHC markers for staging difficult cases of infiltrative BC;
- analyzing markers, determined by inexpensive methods, easily applicable in routine practice in pathology laboratories, useful for classifying UC in molecular classes with prognostic and predictive value.

To achieve the main objectives, we conducted the following studies:

- In the first study, we analyzed the main characteristics of BC cases diagnosed over a period of 5 years in the Pathology Department of the Pius Brînzeu County Emergency Clinical Hospital Timișoara (SCJUPBT).
- In the second study, we analyzed the value of IHC markers smoothelin and smooth muscle actin (SMA) for their use in infiltrative BC cases where the pT1 vs. pT2 classification in TUR specimens was problematic.
- In the third study, we tested the IHC marker panel: GATA3, CK5/6, p16, and FGFR3 as a surrogate method for the molecular classification of MIBC.
- In the last study, we analyzed PD-L1 expression in BC as a potential predictive marker for the immunotherapy response of each molecular class identified using the IHC markers.

Key words: urinary bladder cancer, IHC, smoothelin, SMA, molecular classification, molecular subtypes, GATA3, p16, CK5/6, FGFR3, PD-L1.

GENERAL PART

According to the WHO data from 2020, bladder cancer was the fifth most common type of cancer in Romania, following colorectal, lung, breast, and prostate cancers. The estimated incidence rate (per 100,000 residents) for this neoplasm at the national level is 12.2, and the mortality rate is 3.5. Furthermore, bladder cancer is the ninth leading cause of death from neoplastic diseases in Romania [1]. Bladder cancer predominantly affects elderly men, with the average age at diagnosis being 65-70 years [4].

The right classification of the primary bladder tumor in terms of the pT parameter is crucial for assessing prognosis and determining the appropriate therapeutic management for patients with this neoplasm [5]. The diagnosis of tumor depth invasion in the bladder wall is mainly done on TUR-BT specimens. If the tumor infiltrates the lamina propria (LP) (pT1), the patient is most likely a candidate for conservative tumor treatment, while a clear diagnosis of muscularis propria (MP) invasion (pT2) represents a standard recommendation for aggressive therapy, often radical cystectomy (CR) [5-7]. However, there are situations where diagnostic errors may occur due to the difficulty in differentiating hyperplastic muscularis mucosae (MM) and MP or desmoplastic stroma from MP. To avoid these situations, various IHC markers have been tested to differentiate the two structures (MM and MP), with varied results published in the literature [8-17].

The molecular classification of MIBC, developed using methods like those used for breast cancer, include two major classes (basal and luminal) and were later expanded to six classes, according to a study published in 2020 [18]. These classes, with different histological features, oncogenic mechanisms, and clinical outcomes, partially retain the characteristics of previous classifications and introduce new classes. A current goal in genitourinary pathology is to establish a unique, universal, and standardized panel of IHC markers for the identification of MIBC molecular classes and to integrate it into routine histopathological diagnosis.

Immune checkpoint inhibitors are included as neoadjuvant immunotherapy and are approved as monotherapy or in combination with chemotherapy in the treatment of UC)[19]. IHC testing of PD-L1 is necessary for selecting patients eligible for ICI treatment [20], but the variability in the results regarding the IHC expression of PD-L1 in different clinical studies make it difficult to introduce these tests into everyday practice. The differences in the results of these studies are related to different antibody clones, different automated platforms used for staining, and the variability of interpretation and quantification algorithms for the IHC reaction (expression in immune and/or tumor cells) [3].

SPECIAL PART

1. CLINICO-PATHOLOGICAL CORRELATIONS IN PRIMARY URINARY BLADDER CARCINOMAS

This retrospective study aimed to analyze bladder cancer cases diagnosed and/or treated in a hospital with a high case load for this pathology, over a period of 5 years, from the perspective of the main clinical-demographic and histopathological parameters.

Materials and methods

Cases of UBC diagnosed between January 2016 and December 2020 were selected from the digital archive of the Pathology Department of the SCJUPBT. Recurrent or secondary bladder tumors were excluded. The histological variant of UC and grade were established according to the WHO classification, 4th edition (2016) [4], while pathological staging was determined in accordance with the AJCC TNM system, 8th edition [7].

The study was divided into two parts. The first part included the evaluation of general parameters (clinical and demographical) and the distribution of UBC cases over the years, while the second part was devided into two subgroups:

- Subgroup I included UBC cases diagnosed on biopsy and TUR-BT specimens.
- Subgroup II included cases in which histopathological diagnosis was established on resection specimens.

Cases for which we found in the database of SCJUPBT a diagnosis based on a biopsy or TUR-BT specimen before surgical resection, we performed a comparative analysis of the morphopathological parameters between the two specimen types (biopsy/TUR-BT vs. resection specimen).

Results

We identified 821 cases of BC diagnosed in the Pathology Department of the SCJUPBT. The analysis of the distribution of BC cases according to age group and patient's sex showed that most patients diagnosed with this neoplasm were aged between 60-69 years (33.37%) and 70-79 years (32.52%). Only some cases were observed in the age groups 20-29 years (0.24%) and 30-39 years (1.21%). Regardless of the age group analyzed, the number of male patients was consistently higher than that of female patients. The highest incidence of bladder tumors in men was observed in the age group 60-69 years (27.28%), while for women, the age group 70-79 years (6.33%) showed the highest number of cases. Subgroup I included 737 cases of bladder cancer diagnosed on biopsy and TUR-BT, of which 7 cases (0.95%) were diagnosed in biopsy specimens and 730 cases (99.05%) in TUR-BT specimens. Most cases were UC (99.05%). From the pTNM classification perspective, most tumors (40.30%) were classified as pTa, followed by pT1 tumors (38.26%) and pT2 tumors (18.18%). We identified 7 cases (0.94%) of non-urothelial BC: 3 cases of small cell neuroendocrine carcinoma (NEC), 3 cases of squamous cell carcinoma (SCC), and 1 case of clear cell adenocarcinoma (ADK). Regarding the total number of cases included in subgroup I of the study (n=737), we noticed that in most cases, were conventional UC NMIBC (64.99%) and did not present lymphovascular invasion (LVI) (69.6%), perineural invasion (PNI) (71.64%), or associated carcinoma in situ (CIS) lesions (64.17%). A smaller number of cases were classified as MIBC (18.19%). These tumors predominated in males, all were high-grade (HG), and most cases were UC with divergent differentiation (8.27%). In the MIBC group, the presence of LVI (78%) and PNI (2.44%) was noted at a higher percentage than in the NMIBC group (LVI: 2.44% and PNI: 0.14%). Lymphovascular invasion (LVI) was a parameter of aggressiveness identified exclusively in HG tumors (68 cases - 9.23%). We identified 13 cases (1.76%) of TUR-BT/biopsy that presented both LVI and PNI features and 42 cases (5.70%) where LVI and concurrent CIS lesions were reported.

Subgroup II consisted of 84 BC diagnosed on surgical resection specimens. Tumor invasion beyond the bladder wall was most frequently observed in cases of UC with divergent differentiation (38.09%). In conventional UC, a significantly larger number of cases with tumor invasion limited to the bladder wall (19.04%) were observed. Perineural invasion was predominantly identified in cases of UC with divergent differentiation (34.52%), and most cases where surgical resection margins were positive belonged to the group of UC with divergent differentiation (10.71%). In all tumors classified as NEC/SCC, tumor invasion extended beyond the bladder wall, and PNI aspects were identified. Three out of the four cases of NEC/SCC (3.57% of the total tumors analyzed) had positive surgical resection margins. Considering the traits with an impact on lymph node status, we noted that most pN+ cases were pT3a-pT4b tumors (41.43%) and more frequently associated with LVI (42.86%) and PNI (38.57%). We identified 45 cases (53.57%) of bladder cancer that underwent cystectomy (subgroup II) and had previous TUR-BT performed at SCJUPBT. Among these, 60% maintained their histological variant unchanged, while in 40% of cases, the diagnosis established on the cystectomy specimen was different. In 15.55% of cases, there was a concordance between the pT parameter established in the TUR-BT specimen and the one established on the cystectomy specimen.

2. ASSESSMENT OF SOME IMMUNOHISTOCHEMICAL MARKERS INVOLVED IN STAGING OF UROTHELIAL CARCINOMA DIAGNOSED IN TRANSURETHRAL RESECTION SPECIMENS OF THE URINARY BLADDER

The aim of this study was to analyze the value and applicability of the smoothelin marker in the staging process of UBC in TUR-BT specimens. The study primarily aimed to standardize the smoothelin marker determination method, and then investigate its expression and value in different circumstances.

Materials and methods

I conducted three studies: one prospective and two retrospectives. The prospective part of the study involved collecting three nontumor bladder wall fragments per case from 9 cystectomy specimens. From 12 colectomy cases, one nontumor colon section was selected. For the retrospective part of the study, I used the electronic database of the SAP of SCJUPBT. In the first retrospective study (cases from 2017), I searched for all cases of partial cystectomy, cystoprostatectomy, or pelvic exenteration where the diagnosis of UC with clear MP invasion had been previously established on TUR-BT specimens that were also performed at SCJUPBT. From these, I selected 12 TUR-BT cases. In the second retrospective study, I searched and selected 33 cases of BC diagnosed on TUR-BT specimens (cases from 2017-2018) with equivocal tumor invasion depth.

The following primary antibodies were used: anti-smoothelin (R4A; dilution 1:100; Thermo Fisher; Waltham, MA USA) and anti-SMA (HHF35; RTU; Cell Marque; The Hague, NL). We used heat-induced epitope retrieval (HIER) - Retrieval 1 solution (Bond Epitope Retrieval Solution 1 – ERS1), pH 6.0. Different dilutions of the concentrated smoothelin antibody were evaluated, and a 1:100 dilution was chosen. Nontumor colon and bladder wall sections containing adequate MP were used as positive controls for the smoothelin marker, while vascular smooth muscle cells were used as internal controls for SMA.

Both smoothelin and SMA immunoreactiviry were evaluated in a semi-quantitative manner, following the model suggested by Paner and colleagues [9]. I assessed the reaction intensity (absent, weak, moderate, and strong) and its extent (absent <5% marked muscle cells; focal = positive reaction in 5% - 10% marked muscle cells; heterogeneous = 11% up to 50% marked muscle cells and diffuse >50% marked muscle cells). I used a score that considers both the percentage of positive cells and the staining intensity, as follows: 1 (+): any weak or focal staining; 2 (+): moderate and heterogeneous or diffuse staining, or strong staining but heterogeneous; and 3 (+): strong and diffuse staining. Sections that included complete bladder wall resection, the IHC interpretation of the staining pattern mainly targeted the inner muscle layer of the MP, as these are the smooth muscle bundles typically included in TUR-BT specimens.

Results

The expression of smoothelin and SMA in nontumor urinary bladder and colon sections (positive control)

The smoothelin expression in nontumoral bladder wall sections were as follows: the MM was negative in 8/14 cases (57%), and a staining score of 1+ was observed in 6/14 cases (43%); smoothelin expression in the MP had a score of 1+ in 2/14 cases (14%), 2+ in 7/14 cases (50%), and 3+ in 5/14 cases (36%). When comparing the two components on the same slide, we noted that smoothelin stained the MP stronger in intensity when compared to the staining intensity of MM, in all 14 cases. The SMA staining of the MM in nontumoral bladder wall fragments had a score of 2+ in 4/14 cases (29%) and 3+ in 10/14 cases (71%); for the MP, the following scores were found: 2+ in 5/14 cases (36%) and 3+ in 9/14 cases (64%). In almost all cases, the SMA expression presented a similar intensity and staining pattern in both MM and MP ($r = 0.9859$; $p=0.014$), with a higher staining intensity compared to the staining for smoothelin.

Regarding the 12 cases of nontumoral colon sections, smoothelin was negative in the MM of 6 cases (50%), and in the other 6 cases (50%), a score of 1+ was noted. Concerning the MP, the staining scores for smoothelin were 1+ in 6 cases (50%), 2+ in 3 cases (25%), and 3+ in 3 cases (25%). Both the inner and outer layers of the MP in all 12 cases of nontumoral colon expressed homogeneous staining for this IHC marker. The difference in staining intensity for smoothelin between the MM and MP was always present and in favor of the muscle bundles of the MP ($p=0.001$). Vessels of the LP did not show reactivity for smoothelin.

The staining score for SMA in the case of MM in nontumoral colon sections was 2+ in 5 cases (42%) and 3+ in 7 cases (5%), and for the MP, a staining score of 2+ was observed in 8 cases (67%) and 3+ in 4 cases (33%).

Evaluation of tumor depth invasion using smoothelin and SMA marker in cases of TUR-BT with unequivocal staging of depth invasion

The following results were obtained for smoothelin in the MM: negative in 8/12 cases (67%) and 1+ in 4/12 cases (33%). The smoothelin expression in the MP had a score of 1+ in 4/12 cases (33%), 2+ in 7/12 cases (58%), and 3+ in 1/12 cases (9%). The staining of the vessels in the LP had a score of 1+ in 4/12 cases (33%), and 8/12 cases (67%) showed no immunoreactivity. In 1/12 cases (9%) of TUR-BT, we observed MP bundles with cautery artifacts in which the smoothelin expression in muscle fibers was preserved (2+).

In one of the 12 cases (9%), SMA had an IHC expression score of 2+ in both MM and MP. The other 11 cases (91%) showed diffuse and strong immunoreactivity for SMA in both MM and MP.

The HE slides of 7 from the 12 TUR-BT cases (58%) presented some areas of tumor-infiltrated fragments which were difficult to interpret. Desmoplastic stromal reaction could not be differentiated from the tumor infiltration of the MP. The IHC evaluation of those areas showed a negative reaction for smoothelin and strong, diffuse staining for SMA. The same 7 TUR-BT cases which were stained with smoothelin also showed concomitant IHC expression scores in the unequivocal MP bundles on other resected chips as follows: 1+ in 2 cases, 2+ in 4 cases, and 3+ in one case, and for MM the following scores: negative in 5 cases and 1+ in 2 cases.

Smoothelin and SMA expression in cases of bladder tumors diagnosed on TUR-BT specimens with equivocal staging

We analyzed TUR-BT chips without tumor infiltration, where possible, from the same case and identified 16/33 cases (48%) with hyperplastic MM, which expressed smoothelin as follows: 4/16 - negative, 9/16 - 1+, and 3/16 - 2+. Cases with non-hyperplastic MM (17/33 - 52%) had similar IHC expressions: 6/17 negative, 9/17 - 1+, and 2/17 - 2+. In 4/33 cases (12%), MP was not identified. In the other 29/33 cases (88%), MP presented the following smoothelin staining scores: 3+ in 14/29 cases (48%), 2+ in 12/29 cases (41%), and 1+ in 3/29 cases (10%). Three TUR-BT cases with tumors located in the trigone showed 2+ expression of the smoothelin marker in the superficial muscle fibers. As a result, these were classified as MP. In none of the cases did smoothelin show a higher staining intensity in MM compared to MP. To distinguish between MM and MP, smoothelin had a sensitivity of 100% and a specificity of 53% (p=0.048).

I did not observe any notable changes in the smoothelin staining pattern in the 3/33 (9%) cases of TUR-TV specimens that showed cautery artifacts in the muscle fibers of tumor-infiltrated or nontumor-infiltrated MP.

Over half of the TUR-BT cases - 21/33 (64%) had a 1+ score for smoothelin of the vessel walls in the LP. The other 12/33 cases (36%) showed no staining of the vessels of the LP. The correlation coefficient (r=0.9604) showed a statistically significant positive correlation (p=0.009) between smoothelin staining in MM and that in the smooth muscle fibers of the vessels in TUR-BT specimens with equivocal invasion. Staining for SMA was reported in both MM and MP in the analyzed group.

In 16/33 cases (48%) with equivocal depth of invasion, the difficulty in determining this parameter on HE-stained slides was due to the inability to differentiate dense desmoplastic stroma from MP invasion. IHC analysis of these cases showed SMA staining with a score of 2+ in 3 cases and a score of 3+ in 19 cases, while the smoothelin staining was absent in these areas. In these cases, the staining of smoothelin of the MP from different TUR-BT chips was reported as follows: 1+ expression in one case, 2+ in 5/16 cases, 3+ in 8/16 cases; 2 cases did not show MP (in both cases, smoothelin immunostaining in MM expressed a score of 1+).

The other equivocal cases raised diagnostic issues because the distinction of hyperplastic MM from MP (9/33 - 28%) was problematic due to the unknown origin of thin bundles of smooth muscle fibers dispersed by the tumor (5/33 - 15%), or because of thermally induced artifacts in muscle fibers (3/33 - 9%). All these aspects were analyzed and interpreted based on the performed IHC reactions, and in accordance with the results obtained, the pT parameter was re-established. Thus, after the IHC evaluation of TUR-TV specimens with equivocal invasion on H&E staining, 11 cases (33%) were classified differently from the initial classification regarding the pT parameter.

3. ASSESSMENT OF IMMUNOHISTOCHEMICAL MARKERS WITH POTENTIAL VALUE FOR IDENTIFYING MOLECULAR SUBTYPES OF MUSCLE-INVASIVE UROTHELIAL CARCINOMAS

The objectives for this chapter were as follows: to analyze the IHC marker panel proposed by Allory (part of the EAU group) [18] at the ECP 2020 congress, as a surrogate for molecular determinations, for the purpose of establishing molecular classes of MIBC; and to evaluate the clinicopathological aspects and PD-L1 expression in MIBC in relation to these classes.

Materials and methods

In the first part of the study, we selected 50 consecutive cases of TUT-BT with MIBC from the SCJUPBT archive. In the second part of the study, we performed IHC staining of tumor sections using the IHC autostainer Leica BOND Max (Leica, Wetzlar, Germany), according to the recommended protocols. The following antibodies were used: anti-GATA3 (L50-823; Cell Marque; The Hague, NL), anti-CK5/6 (D5 & 16B4; Cell Marque; The Hague, NL), anti-p16 (6H12; Leica Biosystems; Newcastle, UK), anti-FGFR3 (B-9; Santa Cruz Biotechnology; Dallas, TX, USA), and anti-PD-L1 (28-8, Abcam, Cambridge, UK). In the third part of the process, cases were scanned with the Leica Aperio AT2 slide scanner (Leica Biosystems, Vista, CA, USA) and then transferred to a secure database. Virtual slides were viewed and interpreted for the study using Aperio ImageScope software, version 12.4.3 (Leica Biosystems, Vista, CA, USA).

For the markers GATA3, CK5/6, p16, and FGFR3, the immunoreaction was considered positive if ≥10% of tumor cells showed immunostaining (cut-off of 10%), following the model described by Bontoux et al. [21]. Considering the heterogeneity of CVU, with variations in histological appearance within the same tumor, for each case, we calculated the

average value of the percentage of stained tumor cells, resulting from the evaluation of 3 different areas of the tumor [22].

Expression of PD-L1 in tumor cells was evaluated according to the literature recommendations for 28-8 clone [3,23,24], which was used in the present study. The interpretation was carried out by calculating the ratio between the number of positive tumor cells and the total estimated number of tumor cells on section x100, in the form of a tumor proportion score (TPS), reported as a percentage (%). We used a cut-off of 1% to separate PD-L1-positive tumors (≥1% stained cells) from PD-L1-negative tumors, following the model used in other studies [3,24]. We considered the partial or complete membranous staining of tumor cells, regardless of the staining intensity. We did not evaluate the expression of the marker in nontumor cells and immune cells (lymphocytes, macrophages, dendritic cells, etc.). For the correct interpretation of immunostaining, we considered the morphological characteristics of the cells, evaluated in comparison with the HE-stained slide.

The final step was to quantified the results of the IHC reactions and establish the molecular subtypes according to this scheme: LumP (GATA3 +, CK5/6 +/-, p16 -, FGFR3+); LumU (GATA3 +, Ck5/6 -, p16+ , FGFR3 -); LumNS (GATA3 +, CK5/6 +/-, p16 +, FGFR3 +); Ba/Sq (GATA3 -, CK5/6 +, p16 +/-, FGFR3 +/-) și NE-like (GATA3 -, CK5/6 -, p16 -, FGFR3 -).

Results

The 50 analyzed cases were classified as follows: 23 cases (46%) UC; 18 cases (36%) UC with divergent differentiation, from which - 10 (20%) with squamous differentiation, 5 (10%) with glandular differentiation, and 3 (6%) showing both; 4 cases (8%) nested UC; 2 cases (4%) of micropapillary UC; 2 cases (4%) of plasmacytoid UC, and 1 case (2%) of lymphoepithelioma-like UC. There were 43 cases (86%) with GATA3+/CK5/6- IHC profile, classified as luminal tumors, and 7 cases (14%) with GATA3-/CK5/6+ profile, classified as basal tumors. According to the expression of the four IHC markers used, the cases were grouped in molecular subtypes as follows: 17 tumors (34%) LumP, 14 tumors (28%) LumU, 12 tumors (24%) LumNS, and 7 tumors (14%) Ba/Sq. All LumP-classified cases (17/17 -100%) were GATA3 and FGFR3 positive and p16 negative. Of the 17 LumP cases, 11 cases (64.7%) showed moderate (6 – 54.54%) or intense (5 – 45.45%) expression of the CK5/6 marker in tumor cells. All LumU cases (14/14 – 100%) expressed GATA3 and p16 and were negative for CK5/6 and FGFR3. Of the 12 LumNS cases, 7/12 (58.33%) were positive for GATA3, p16, and FGFR3 and negative for CK5/6, and the other 5/12 cases (41.66%) were positive for all four markers analyzed. All Ba/Sq-classified cases (7/7 – 100%) were CK5/6-positive and GATA3-negative. Of the 7 Ba/Sq-classified cases, 3/7 cases (42.85%) were FGFR3-positive, 2/7 cases (28.57%) were p16-positive, 5/7 cases (71.42%) were p16-negative, and 4/7 cases (51.14%) were FGFR3-negative. Twelve of the 50 tumors (24%) analyzed showed PD-L1 staining of tumor cells, with a membranous staining pattern of variable, weak, or moderate intensity.

The clinical and pathological characteristics associated with each IHC determined molecular subtype

The association of IHC determined molecular subtypes with histological variants of UC showed the following distribution of LumP cases: 8/17 (47.05%) conventional UC, 4/17 (23.52%) UC with squamous differentiation, 2/17 (11.76%) UC with squamous and glandular differentiation, 1/17 (5.88%) UC with glandular differentiation, and 2/17 (11.76%) nested UC. Regarding the LumU-classified cases, they included: 7/14 (50%) conventional UC, 2/14 (14.28%) UC with glandular differentiation, 1/14 (7.14%) %) UC with squamous differentiation, 2/14 (14.28%) micropapillary UC, 1/14 (7.14%) lymphoepithelioma-like UC, and 1/14 (7.14%) plasmacytoid UC. Of the 12 LumNS cases, 6/12 (50%) were conventional UC, 2/12 (16.66%) nested UC, 1/12 (8.33%) plasmacytoid UC, 1/12 (8.33%) UC with squamous differentiation, 1/12 (8.33%) UC with glandular differentiation, and 1/12 (8.33%) UC with squamous and glandular differentiation. Of the Ba/Sq cases, most were UC with squamous differentiation - 4/7 (57.14%) and only 2/7 (28.57%) were conventional UC, respectively 1/7 (14.28%) UC with glandular differentiation.

PD-L1 expression and the molecular subtypes of MIBC

The PD-L1-positive tumors consisted of 6 conventional UC (50%), 3 (25%) UC with squamous differentiation, 1 (8.33%) UC with glandular differentiation, 1 (8.33%) nested UC, and 1 (8.33%) lymphoepithelioma-like UC. PD-L1 expression was present in 3/17 LumP tumors (17.64%), in 4/14 (28.57%) LumU tumors, in 1/12 (8.33%) LumNS tumors, and in 4/7 (57.14%) Ba/Sq tumors.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

o Between January 2016 and December 2020, we identified 821 cases of bladder cancer diagnosed in SAP of SCJUPBT, with many patients being male (M:F ratio of 3:1). The seventh decade represented the age range with the highest incidence of bladder cancer among men, and the eighth decade among women. Most bladder cancer cases (>95%) were UC, with conventional UC being the most frequently reported variant in TUR-BT/biopsy specimens, and UC with divergent differentiation in cystectomy specimens.

o Most tumor cases from TUR-BT/biopsy group were NMIBC (80%), characterized by the lack of LVI, PNI, or associated CIS lesions. The correlation analysis showed the association of tumor invasion with all evaluated prognostic and progression factors (tumor grade, histological variant, LVI, PNI, and associated CIS) ($p<0.0001$).

o The analysis of the cystectomy group showed that tumors classified as pTa-pT2 were predominantly conventional UC (19%), most without LVI (15%) or PNI (24%), while tumors classified as pT3-pT4 were predominantly UC with divergent differentiation (38%), with LVI (64%), PNI (64%), and lymph node metastases (41%) ($p<0.05$).

o The results of the study evaluating the IHC markers smoothelin and SMA in the analyzed groups (including non-tumor bladder and intestinal wall fragments and UC samples from TUR-BT specimens with unequivocal and equivocal pT classification) highlighted the specificity of smoothelin for the MP, unlike SMA, for which we observed a strong and diffuse reaction in smooth muscle cells of the MM and MP, as well as in reactive myofibroblasts in the stroma. At the same time, we found that smoothelin expression in the MM was always weaker in intensity compared to that in the MP on the same section/slide, and that no case showed a 3+ score for smoothelin in the MM. No significant differences were identified regarding smoothelin expression between hyperplastic and non-hyperplastic muscularis mucosae.

o Our recommendations for the interpretation of the immunoreaction for smoothelin are as follows: (1) have an internal control showing the MM and MP labeled with different intensities, (2) adopt a high threshold for marker expression in the staining of the MP (for example, a score of +3 or at least a 2-level staining difference compared to marker expression in the MM), and (3) interpret the reaction on well-represented/rich MP bundles, avoiding the interpretation of marker expression on thin/fragmented muscle bundles.

o The study of the IHC markers used for the "molecular" classification of UCB (GATA3, CK5/6, p16, and FGFR3) showed that 86% of cases were classified in the luminal molecular class (GATA3+ and CK5/6-), while 14% were included in the basal molecular class (GATA3- and CK5/6+). Most luminal tumors were classified in the LumP molecular subtype (34%), followed by LumU tumors (28%). Tumors in the LumP subtype most frequently showed PNI, LVI, and associated CIS lesions. The LumU subtype included a larger number of tumors representing other histological variants of UC than the conventional one, including micropapillary UC, lymphoepithelioma-like UC, and plasmacytoid UC.

o From the perspective of therapeutic implications of the molecular classes identified by IHC, we observed that all LumP and LumNS tumors expressed FGFR3. At the same time, out of the 50 cases analyzed, 24% showed positive PD-L1 reaction in tumor cells (TPS $\geq$1%), the majority (57%) being tumors from the Ba/Sq class.

o The importance of the FGFR3 reaction calibration lies in the fact that it could contribute, if a relationship between marker expression and FGFR3 mutations can be established, to the selection of eligible patients for neoadjuvant treatment with the pan-FGFR inhibitor, Erdafitinib, given that patients with advanced MIBC and FGFR2/3 mutations benefit from this treatment in other countries.

o The originality and novelty of the thesis lies in the use of more sophisticated and demanding IHC markers, such as smoothelin, a marker that is very rarely used in pathological anatomy laboratories. To ensure the accuracy and reproducibility of the results, the use of the antibody required rigorous standardization of the working method, carried out before the start of the experimental phase. This objective was achieved by optimizing the processing technique and the method of interpretation and evaluation of the IHC reaction for this marker. To the best of our knowledge, our study is the

only one of its kind in Romania and, according to the results obtained, has practical applicability. Although the number of infiltrative TV cases with uncertain MP invasion evaluated using the anti-smoothelin antibody was limited, the marker proves to be attractive as part of an IHC marker panel, useful for difficult-to-classify cases in terms depth of invasion in TUR-BT specimens, due to its ability to differentiate between MM and stromal desmoplastic reaction and MP.

o Another nationally novel aspect is related to the use of a limited panel of IHC markers (GATA3, CK5/6, p16, and FGFR3), less commonly used internationally for the molecular classification of UCB, even more in a context where the choice of type and number of IHC markers used is controversial and an important topic in terms of the cost-benefit ratio.

o Within the molecular subtypes, we evaluated the expression of PD-L1 and opted for 28-8clone (Leica) due to its financial accessibility and the speed with which the expression can be interpreted by evaluating the TPS. At a time when there are numerous anti-PD-L1 antibody clones and numerous scoring systems for reaction, the 28-8 clone could be considered for selecting UCB cases (PD-L1-positive tumors with TPS ≥1%) that could benefit from anti-PD1/PD-L1 treatments, if it is studied on larger patient cohorts.

LIST OF PUBLICATIONS

1. **Mihai I**, Taban S, Cumpanas A, Olteanu EG, Iacob M, Dema A. Clear cell urothelial carcinoma of the urinary bladder-a rare pathological entity. A case report and a systematic review of the literature. Bosnian journal of basic medical sciences. 2019 Nov;19(4):400. DOI: https://doi.org/10.17305/bjbms.2019.4182 **IF= 2.07**
2. Olteanu GE, **Mihai IM**, Bojin F, Gavriliuc O, Paunescu V. The natural adaptive evolution of cancer: The metastatic ability of cancer cells. Bosnian Journal of Basic Medical Sciences. 2020 Feb 3. DOI: https://doi.org/10.17305/bjbms.2019.4565 **IF= 3.05**
3. **Mihai IM**, Olteanu G-E, Herman D, et al. Analysis of Tumor Depth Invasion With Anti-Smoothelin Antibody in Equivocal Transurethral Resection of Urinary Bladder Tumor Surgical Specimens. International Journal of Surgical Pathology. October 2020. doi:10.1177/1066896920967762 **IF= 1.35**

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