Research Article

Molecular classification using Lund University algorithm and clinical correlations in muscle-invasive bladder cancer: Insights from a retrospective study

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Abstract

Background: Muscle-invasive bladder cancer (MIBC) is universally classified as high-grade urothelial carcinoma, precluding the use of histological grading alone for prognostication. However, specific morphological features of MIBC may provide useful information to guide treatment decisions. In the last decade, there has been increasing interest in genetic profiling of MIBC. **Objective:** The aim of the study is to validate the use of Lund Classification in attributing phenotype to large series with extreme reliability and reproducibility compared to all histological sections in the clinical practice. **Methods:** We performed a molecular profiling study on a large, consecutive cohort of MIBC cases using a straightforward immunohistochemical algorithm aligned with the Lund Classification. **Results:** We evaluated 450 MIBC cases. In a subgroup of 103 patients, we assessed the concordance between transurethral resection of bladder tumor (TURBT) specimens and cystectomy on paired samples. Luminal tumor types showed a statistically significant association with the usual histotype, while basal and NULL types were more frequently associated with variant histotypes ($p < 0.0001$). A stromal lymphocytic infiltrate ≥10% was more commonly observed in basal types ($p < 0.0001$). Basal types also exhibited higher positive rates of human epidermal growth factor receptor-2 (HER2/neu) positivity, while luminal types were more likely to be positive for tumor suppressor protein p53. Luminal types have demonstrated longer survival compared to their basal and NULL counterparts. In the concordance analysis, tumor type assignment based on TURBT showed sensitivity, specificity, and both positive and negative predictive values of 100% for basal and NULL types. The predictive accuracy for luminal types on TURBT ranged between 89.5% and 98.2%. **Conclusion:** Our findings demonstrate the feasibility of applying the Lund Classification for molecular subtyping of MIBC in routine diagnostics. The consistency in tumor type assignment between TURBT and cystectomy samples further supports the clinical utility of this approach. Tumor types significantly influenced survival outcomes, underscoring its relevance in patient stratification and personalized treatment strategies.

Keywords: Bladder cancer, Immunohistochemistry, Lund taxonomy, Molecular subtype, Survival

1. INTRODUCTION

Bladder cancer (BC) is the ninth most common malignancy and the thirteenth leading cause of cancer-related death in Western countries. According to data from the Global Cancer Observatory, 36% of patients diagnosed with BC worldwide will die from the disease [1]. BCs are classified according to various clinical and pathological factors that influence prognosis. The primary distinctions are between non-invasive (or superficial) forms and invasive forms, high-grade and low-grade tumors, and different histological variants.

Muscle-invasive bladder cancers (MIBCs, including T2–T4 disease) account for 30–40% of cases at diagnosis

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[2]. Approximately 30% of MIBC patients are at risk of having micrometastatic disease at diagnosis, with the 5-year survival rate being 60% and 5%, respectively. Neoadjuvant chemotherapy has been developed to improve these outcomes [3,4], although its use can be restricted by the patient's comorbidities. Moreover, MIBC is a heterogeneous disease characterized by a high frequency of mutations and genomic instability, often leading to chemotherapeutic resistance [5].

In recent years, immunotherapy has emerged as a promising alternative due to its better tolerability and fewer adverse effects. However, identifying reliable criteria for selecting patients who will respond better to chemotherapy versus immunotherapy remains challenging. Comprehensive mRNA expression profiles in BC have recently been used to classify MIBCs into various molecular subtypes for more accurate prognostic and therapeutic stratification. The primary molecular subtypes recognized in MIBCs are the luminal and basal subtypes [6]. Several institutions, including the University of North Carolina, MD Anderson Cancer Center, Lund University, and The Cancer Genome Atlas (TCGA), have proposed molecular classifications of MIBCs based on transcriptomic analysis of tumor mRNA [7-12]. These groups have developed a consensus molecular classification, describing six major molecular subgroups, with Basal/Squamous (Ba/Sq), Luminal Papillary (LumP), and Luminal Unstable (LumU) subtypes constituting the majority of cases [13]. However, their use remains limited to clinical studies, partly due to the insufficient availability of biomarkers to select subgroups of patients who would benefit most from specific therapies [14,15].

Currently, these methodologies have yet to be validated in clinical practice [16]. The present study aims to describe a series of MIBC cases from the University Hospital of Parma and characterize them morphologically and immunohistochemically following Lund University's diagnostic classification [8].

2. METHODS

This retrospective study analyzed a series of MIBCs observed at the Pathology Unit of the University Hospital of Parma from 1999 to 2020. Histological slides were reevaluated and reclassified according to current diagnostic criteria [17]. Inclusion criteria were as follows: a primary diagnosis of high-grade (WHO/ISUP) urothelial carcinoma with invasion of the muscularis propria, and the availability of pathological materials from transurethral resection of bladder tumor (TURBT) or cystectomy, sufficient for histopathological evaluation and immunohistochemical studies. Only the immunohistochemical profile of the muscle-invasive component, not the superficial component, was considered for classification purposes.

For each patient, the following clinical data were collected: sex, age, history of urothelial tumors, or other neoplasms, follow-up information, and, if applicable, the date and cause of death. Mortality data were obtained through the Cancer Registry. Causes of death were categorized as tumor-specific or non-tumor-specific. Histopathological evaluation was performed consensually by two pathologists and included the recording of predefined variables: Histotype (usual or variant), presence or absence of perineural invasion (PNI), lymphovascular infiltration (LVI), stromal tumor-infiltrating lymphocytes (stromal TILs), and stage.

MIBCs were immunhistomechemically characterized techniques, on both on tissue microarray (TMA) and whole histological sections. Tissue antigen expression profiles were analyzed according to the classification algorithm proposed by Lund University [18]. This algorithm includes the employment of the following antibodies: GATA-binding protein 3 (GATA3), cytokeratin 5/6 (CK5/6), cytokeratin 14 (CK14), tumor suppressor p16, cyclin D1 (CCD1), vimentin (VIM), and epithelial cell adhesion molecule (EpCAM). For TMA analysis, three 0.6 mm cores were prepared to ensure a better representation of the whole tissue section expression. In cases where sufficient tissue was available, immunoreactivity for human epidermal growth factor receptor-2 (HER2/neu) and tumor suppressor protein p53 was also evaluated. Immunohistochemical reactions were performed using the Ultraview Detection Kit (05907136001, Roche Diagnostics polymeric system, Switzerland), and slides were processed using the Benchmark Ultra (N750-BMKU-FS 05342716001, Roche Diagnostics automatic immunostainer, Switzerland) (Table S1 and Figure S1).

Histotypes were categorized according to current standard practices: luminal urothelial-like (URO), luminal non-specified (NAS), luminal genomically unstable (GU), basal, mesenchymal-like (Mes-like), neuroendocrine-like (NE-like), and null (NULL), as well as variant histotype. Variant histotypes considered included glandular, lymphoepithelioma-like, micropapillary, NE-like, nested, rhabdoid/plasmacytoid, sarcomatoid, squamous, and tubular types. Stromal TILs were estimated in 10% intervals (percentage of stromal area).

For tumors diagnosed solely by TURBT, where extravesical extension could not be defined, the stage was classified as T2+. The staging was performed according to the American Joint Commission on Cancer (AJCC) guidelines into stages II, IIIA, IIIB, and IV (excluding M1) [19]. For statistical analysis, stages IIIB and IV were combined into a single category.

The study was approved by the Ethics Committee of the Emilia Ovest Area on October 22, 2019, protocol number 615/2019/TESS/AOUPR. The primary objectives of the study are to test the reliability and applicability of the Lund Classification algorithm in a real-world context, evaluate the

reproducibility of tumor type assignment between TURBT and cystectomy samples, and assess the impact of tumor types on overall survival (OS) and tumor-specific survival (TSS).

Age was considered as both a continuous and a categorical variable (≤ 60 years, 61–70 years, 71–80 years, > 80 years). Follow-up was defined as the time interval between the date of the first MIBC diagnosis (TURBT) and the date of death or the last patient evaluation (as of April 30, 2022). For statistical purposes, luminal URO and NAS tumor types were combined into a single category, as were Mes-like, NE-like, and NULL types, which were grouped into a category labeled "NULL."

For statistical analysis, quantitative variables are expressed as means and standard deviations (SD). The main correlations were evaluated using contingency tables and the Chi-square test. Statistical significance was considered for $p < 0.05$ with 95% confidence intervals. For paired TURBT and cystectomy data, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Survival analysis was conducted using Kaplan–Meier curves and univariate and multivariate Cox regression.

Various clinical and pathological variables were analyzed to OS and TSS using Cox regression analysis and Kaplan–Meier curves. The variables considered included age (as continuous and categorical variable), sex, type of intervention, presence of histological variants, a history of previous urothelial neoplasms, stromal TILs, stage, and immunohistochemical markers (HER2/neu and p53).

3. RESULTS

3.1. Patient characteristics

A total of 450 samples were analyzed, with the immunohistochemical results summarized in Table S2. Of these, 215 (47.78%) were obtained from TURBT and 235 (52.22%) from cystectomies. Among the patients, 347 underwent either TURBT or cystectomy alone (Group A), whereas 103 patients were evaluated using paired samples from both cystectomy and TURBT (Group B). Immunohistochemical analysis of the pathological samples was conducted on all histological sections for recent cases (249 cases) as part of routine analysis, and on TMA for older samples (201 cases), to preserve tissue for further analyses. No statistically significant differences were found between the two groups with respect to stage ($p = 0.069$), with stage IIIA being the most common in both groups; tumor types ($p = 0.554$), with luminal URO being the most frequent, followed by luminal GU; or the incidence of histotypes, where usual, sarcomatoid, and squamous forms were the most frequent in both groups.

In the entire cohort, tumor types did not show statistically significant correlations with the examined clinical variables (Tables S3 and S4). However, luminal URO-NAS tumor types were more frequently associated with the absence of previous urothelial neoplasms ($p = 0.040$). Luminal tumor types were significantly more likely associated with the usual histotype, while basal and NULL types were more commonly associated with variant histotypes ($p < 0.0001$). A stromal lymphocytic infiltrate ≥10% was observed more frequently in basal tumor types ($p < 0.0001$). No other variables showed significant differences in distribution between the tumor types.

Tumor types were also correlated with two immunohistochemical markers of prognostic and therapeutic significance: HER2/neu and p53 (Table 1). Both correlations were statistically significant, with HER2/neu positivity being more frequent in luminal types ($p = 0.005$) and URO-NAS luminal types exhibiting "wild type" p53 expression ($p < 0.0001$).

In Group B, the concordance between TURBT and cystectomy samples was evaluated. Out of 103 paired cases, only 3 (2.91%) showed discrepancies in tumor type, all of which were luminal tumors. Two cases that were classified as luminal URO-NAS on TURBT were later reclassified as luminal GU upon subsequent cystectomy. A third case was of GU luminal type on TURBT and URO-NAS luminal type on cystectomy. Morphological discordance was also evaluated, with 3 (2.91%) cases showing inconsistent morphology between the TURBT and cystectomy samples: Two NULL and one luminal. The luminal tumor was also immunohistochemically discordant.

In the concordance analysis, the attribution of tumor type on TURBT showed a sensitivity, specificity, and both PPV and NPV of 100% for both basal and NULL tumor types. The predictive capacity for luminal types on TURBT varied between 89.5% and 98.2%, where PPV for URO-NAS was 97.9% and for GU 89.5%, whereas NPV for URO-NAS was 98.2% and GU 97.6%. Higher sensitivity and specificity were observed for URO-NAS luminal types, with a sensitivity of 97.9% (URO-NAS) versus 89.5% (GU) and a specificity of 98.2% (URO-NAS) versus 97.6% (GU).

3.2. Survival analysis

In the both univariate and multivariate analyses of the entire sample, age (considered as both a continuous and a categorical variable), stromal TILs, and stage were significantly correlated with both overall OS and tumor-specific survival TSS (Table 2 and Figure 1). For the purpose of survival analysis, tumor types were divided into three groups: URO-NAS-GU luminal, basal, and NULL. However, no significant differences were observed in OS or TSS between these groups (Table S5 and Figure S1).

The 1-year survival rate from diagnosis was 49.9% for basal tumor types, compared to 74.3% for luminal types and

Table 1. Correlation between immunohistochemically-based subtypes with human epidermal growth factor receptor-2 and tumor suppressor protein p53

Variables	Luminal-like URO-NAS (%)	Luminal-like GU (%)	Basal-like (%)	NULL (%)	*p*-value
HER2/neu					
Neg (score 0, 1+)	62 (17.87)	23 (6.63)	32 (9.22)	26 (7.49)	0.005*
Pos (score 2, 3+)	15 (4.32)	8 (2.31)	1 (0.29)	0	
N/A	3 (0.86)	2 (0.58)	0	0	
p53					
MUT (overexpressed or null)	22 (6.34)	25 (7.20)	28 (8.07)	17 (4.90)	<0.0001*
WT	53 (15.27)	6 (1.73)	5 (1.44)	7 (2.02)	
N/A	5 (1.44)	2 (0.58)	0	2 (0.58)	

Note: *indicates statistical significance.

GU: Genomically unstable, HER2/neu: Human epidermal growth factor receptor-2, MUT: Mutated phenotype, NAS: Non-specified, Neg: Negative, N/A: Not assessable, Pos: Positive, p53: Tumor suppressor protein p53, URO: Urothelial-like, WT: Wild type.

Table 2. Univariate survival analysis and Cox regressions for overall and tumor-specific mortality

Variable	OS			TSS		
	HR	CI (95%)	*P*-value	HR	CI (95%)	*p*-value
Age (in years)						
Continue (per year)	1.05	1.03–1.06	<0.0001*	1.04	1.03–1.06	<0.0001*
>80	-			-		
71–80	0.51	0.36–0.72	<0.0001*	0.60	0.41–0.86	<0.0001*
61–70	0.29	0.19–0.44	<0.0001*	0.35	0.22–0.55	<0.0001*
≤60	0.24	0.14–0.42	<0.0001*	0.25	0.14–0.46	<0.0001*
Sex						
Males	-			-		
Females	1.08	0.80–1.46	0.608	1.09	0.79–1.51	0.589
Procedure						
TURBT	-			-		
Cystectomy	0.92	0.67–1.26	0.605	1.04	0.73–1.47	0.846
Histological subtypes						
No	-			-		
Yes	1.12	0.84–1.51	0.443	1.12	0.81–1.55	0.486
Medical history of urothelial tumor						
No	-			-		
Yes	0.94	0.64–1.38	0.750	0.98	0.65–1.47	0.905
Stromal TILs						
>10%	-			-		
≤10%	1.98	1.37–2.85	<0.0001*	2.17	1.44–3.27	<0.0001*
AJCC group staging						
II	-			-		
IIIA	2.51	1.54–4.09	<0.0001*	2.72	1.58–4.68	<0.0001*
IIIB/IV	3.06	1.69–5.57	<0.0001*	3.30	1.71–6.33	<0.0001*
HER2/neu						
Neg (score 0,1+)	-			-		
Pos (score 2,3+)	1.07	0.65–1.75	0.789	1.19	0.71–2.01	0.511
p53						
WT (wild type)	-			-		
MUT (overexpressed/null)	1.24	0.87–1.78	0.237	1.15	0.78–1.70	0.492

Note: *indicates statistical significance.

AJCC: American Joint Commission on Cancer, CI: Confidence interval, HER2/neu: Human epidermal growth factor receptor-2, HR: Hazard ratio, OS: Overall survival, Pos: Positive, p53: Tumor suppressor protein p53, MUT: Mutation, Neg: Negative, TILs: Tumor-infiltrating lymphocytes, TSS: Tumor-specific survival, TURBT: Transurethral resection of bladder tumor.

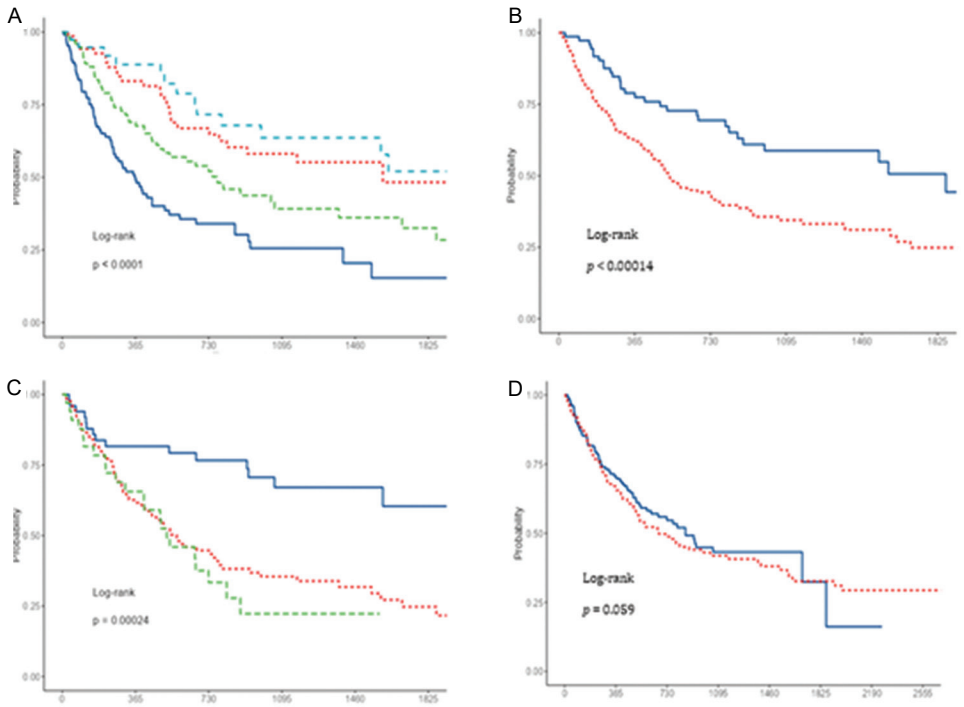

Figure 1. Kaplan–Meier tumor-specific survival curves: Age in classes (A), stromal TILs (B), group staging according to the AJCC (C), and sex (D). Notes: Panel A: Blue = >80 years old; green = 71–80 years old; red = 60–70 years old; light blue = ≤60 years old. Panel B: Red = Stromal TILs ≤10%; blue = Stromal TILs >10%. Panel C: Green = Stage group IIIB/IV; red = Stage group IIIA; blue = Stage group II. Panel D: Red = Males; blue = Females. Time data on the X-axis are expressed in days.
AJCC: American Joint Commission on Cancer, TIL: Tumor-infiltrating lymphocytes.

73.4% for NULL types. At 3 years, the survival rate for basal tumor types was 37.6%, whereas for luminal and NULL types, it was 42.0% and 58.3%, respectively. At 5 years, the survival rate for basal types remained 37.6%, whereas it was 32.2% for luminal types and 41.6% for NULL types.

Variables found to be statistically significant in univariate analysis or deemed biologically relevant were used in multivariate analysis models to evaluate their independent effect on survival. One such model included age (considered a continuous variable), AJCC stage, stromal TILs, and tumor type (categorized into three classes) (Table 3). This model confirmed that age, AJCC stage, and stromal TILs were independently associated with survival outcomes. In terms of tumor type, after controlling for confounding variables, an independent effect was observed but was not apparent in the univariate analysis. Specifically, luminal types showed a 35% reduction in mortality compared to basal types, and NULL types exhibited a 36% reduction in mortality versus basal types.

To further explore the relationship between stromal TILs and tumor type, we combined these two variables and divided them into four groups: basal/TILs-, basal/TILs+, luminal/TILs-, and luminal/TILs+. The results of the tumor-specific univariate survival analysis for this derived variable are shown in Tables S3 and S4. This combination resulted in a highly significant stratification between the groups (log-rank, $p < 0.0001$). The 1-year survival rates for the extreme groups (basal/TIL− vs. luminal/TILs+) were 39.4% and 92.4%,

Table 3. Tumor-specific multivariate survival analysis: Immunohistochemically-based tumor subtyping (three classes), stromal lymphocytic infiltrates, group staging according to the American Joint Commission on Cancer and Age

Variable	HR (CI 95%, *p*-value)
Stromal TILs	
>10%	-
≤10%	2.48 (1.61–3.84, <0.0001*)
IHC-based subtype	
Basal-like	-
Luminal-like URO-NAS-GU	0.65 (0.43–0.97, 0.037*)
NULL	0.64 (0.35–1.16, 0.139)
AJCC group staging	
T2+	-
IIIA	1.66 (1.09–2.52, 0.018*)
II	0.71 (0.38–1.31, 0.267)
IIIB/IV	2.31 (1.27–4.20, 0.006*)
Age	
-	1.05 (1.03–1.07, <0.0001*)

Note: *indicates statistical significance.
AJCC: American Joint Commission on Cancer, CI: Confidence interval, GU: Genomically unstable, HR: Hazard ratio, IHC: Immunohistochemistry, NAS: Non-specified, TILs: Tumor-infiltrating lymphocytes, URO: Urothelial-like.

respectively, and the 3-year survival rates were 26.3% and 65.5%, respectively. In a multivariate model controlled for age and AJCC stage, all four derived groups showed a statistically significant independent effect, with good risk stratification across the groups (Table 4).

4. DISCUSSION

A more precise characterization of MIBCs in terms of subtypes based on their gene expression is essential for better risk stratification and therapeutic management. Such classifications can define the biological profiles of these tumors and predict their responses to various treatments.

The first major subdivision of MIBCs was proposed by the University of North Carolina in 2014, distinguishing between basal and luminal subtypes based on the differential expression of genes associated with normal urothelial differentiation. This differentiation is clinically significant since basal MIBCs tend to be more aggressive and have a higher propensity for metastasis [20]. However, a dichotomous model that separates MIBCs into just luminal and basal types does not fully capture the considerable clinical and pathological heterogeneity of the disease.

The work of TCGA provided a more integrated analysis by using various "-omic" platforms (genomics, transcriptomics, proteomics, and methylomics), which offered a comprehensive molecular characterization of MIBC. This approach advanced our understanding of the clinical and pathological implications of molecular defects and identified potential therapeutic targets [10,11]. TCGA's findings revealed that bladder urothelial carcinoma is a high-mutation neoplasm, with a median of 5.5 mutations per megabase. This study highlighted that 69% of MIBCs possess potential therapeutic molecular targets specific to each subtype, identifying six molecular subtypes of MIBC: LumP, Luminal Non-specific (LumNS), LumU, stromal-rich, Ba/Sq, and NE-like [21,22].

Although the dichotomous luminal-basal model has been expanded upon by TCGA, the high costs and technological complexity of TCGA-based analysis have limited its widespread clinical application. As a result, several groups have proposed alternative, more accessible immunohistochemical algorithms using a limited number of gene expression markers to classify MIBC [23,24].

Lund University developed an immunohistochemical algorithm based on the expression of two markers, GATA3 and CK5/6, to classify MIBC into luminal and basal subtypes, respectively. Additional markers such as CCD1 for luminal URO, p16 for luminal GU, CK14 for basal-squamous, VIM for Mes-like, and EpCAM for NE-like subtype further refine the classification [25]. According to this algorithm, five tumor types are distinguished: Luminal URO, luminal GU, basal, Mes-like, and NE-like [26]. This classification system is applicable to both endoscopic biopsies (TURBT) and surgical specimens (cystectomies), as well as to non-muscle-invasive BCs and MIBCs [27,28].

In our study, we conducted a morphological and immunohistochemical analysis of 450 MIBC cases, including 103 cases that were examined using paired TURBT cystectomy samples. These paired samples were used to assess the reliability of tumor classification based on endoscopic biopsies relative compared to the cystectomy specimens. In contrast to our series, Marzouka *et al.* [29] applied the Lund algorithm to TCGA cases and observed an overrepresentation of luminal URO types and an underrepresentation of luminal GU types (luminal URO 42% vs. 34%, luminal GU 14% vs. 23.3%, basal 27% vs. 21.5%, Mes-like 9% vs. 5.7%, NE-like 5% vs. 4.9%, and NULL 3% vs. 2%). This discrepancy may stem from sample selection and the multicenter nature of the TCGA cohort. In our study, tumor type classification was consistent in 100% of basal, luminal, and NULL cases. Among the luminal tumors, only 7.78% were unclassifiable (luminal NAS) as either luminal URO or luminal GU. We found a correlation between tumor types and a negative history of urothelial neoplasms in luminal forms (50.15% for luminal vs. 30.55% for non-luminal, $p = 0.040$). In addition, variant histology was prevalent in basal, and NULL types (23.06% for basal+NULL vs. 14.13% for luminal, $p < 0.0001$), and the presence of stromal TILs (≥10%) was also predominant in basal and NULL types (23.06% for basal+NULL vs. 14.13% for luminal, $p < 0.0001$). Tumor types did not correlate with age or AJCC stage at diagnosis, which contradicts findings by Kamoun *et al.* [13], who observed a higher frequency of pT2 stage and younger age (under 60 years) in luminal

Table 4. Tumor-specific multivariate survival analysis adjusted for age and the AJCC group staging: immunohistochemically-based subtype and stromal TILs

Variable	HR (IC 95%, *p*-value)
IHC-based subtype	
Basal-like/stromal TILs−	-
Luminal-like/stromal TILs−	0.65 (0.42–1.00, 0.050*)
Basal-like/stromal TILs+	0.44 (0.23–0.84, 0.014*)
Luminal-like/stromal TILs+	0.23 (0.12–0.45, <0.0001*)
AJCC group stage	
T2+	-
IIIA	1.74 (1.18–2.57, 0.006*)
II	0.66 (0.36–1.18, 0.161)
IIIB/IV	2.73 (1.57–4.73, <0.0001*)
Age	
-	1.05 (1.03–1.07, <0.0001*)

Note: *indicates statistical significance.

AJCC: American Joint Commission on Cancer, CI: Confidence interval, HR: Hazard ratio, IHC: Immunohistochemistry, TILs: Tumor-infiltrating lymphocytes.

types. Another study has reported that basal types are more commonly found in the pT3/T4 stage and older patients [21].

Our survival analysis showed that age ($p < 0.0001$), AJCC stage ($p = 0.006$), and poor lymphocytic response (TILs) ($p < 0.0001$) were all significantly correlated with a higher risk of both OS and TSS in univariate and multivariate analyses. The effect of age and stage on OS is well-supported by previous literature [20]. The role of stromal TILs in survival has been extensively studied in other cancers (such as breast, lung, and colorectal) [30], where a poor lymphocytic response is generally associated with a worse prognosis, as we observed in this study. However, the prognostic significance of TILs in MIBC remains debated [31]. Regarding the effect of tumor type, we found that basal tumor types were associated with higher tumor-specific mortality compared to non-basal types in both univariate ($p = 0.044$) and multivariate ($p = 0.030$) analyses. This finding contradicts other studies suggesting that luminal types are a significant predictor of tumor-specific mortality [32], likely due to the heterogeneity in study populations and statistical analyses. Nevertheless, the greater negative impact of basal types on OS is widely demonstrated [33], which makes this discrepancy noteworthy.

Combining tumor type and stromal TILs into four risk groups provided an effective risk stratification, with a mortality difference of 53% and 39.2% at 1 and 3 years, respectively, between the extreme groups (basal/TILs− vs. luminal/TILs+). This novel finding could have significant clinical implications, especially given the lack of other prognostic histopathological markers in MIBCs, aside from stage.

Regardless of the type of tumor sampling, the distribution of observed tumor types was comparable (Table S4). In the direct comparison between paired TURBT and subsequent cystectomy cases, it was demonstrated that the attribution of tumor type based on diagnostic biopsy is highly reliable, with a concordance rate of 97%. For luminal tumor types, sensitivity ranged from 89.5% to 97.9%, while specificity ranged from 97.6% to 98.2%. For basal and NULL types, both sensitivity and specificity were 100%. The predictive power of TURBT, when compared to cystectomy, along with the prognostic impact of TILs, supports the use of these parameters to guide clinical management more accurately.

Basal tumor types have been shown to be more responsive to neoadjuvant chemotherapy [21,34] and are potential candidates for immunomodulatory drugs, such as atezolizumab [22]. On the other hand, luminal types may be more suitable for therapies targeting fibroblast growth factor receptors 3 [13]. A retrospective Swedish study [35] did not highlight a significant impact of tumor types on tumor-specific mortality. The discrepancy with our results may stem from differences in population characteristics, risk factors, and tumor typing methods (molecular vs. immunohistochemical). Another factor to consider in explaining this difference is the lack of information regarding neoadjuvant or adjuvant treatments in our cohort. This is especially relevant, given that only 15% of patients in the Swedish study received chemotherapy, either as neoadjuvant/induction or adjuvant therapy. A classification approach based on immunohistochemical profiles, rather than gene expression profiles, allows for verification of the spatial relationship between marker expression and the tumor. Conversely, gene expression profiles from tissue extracts cannot separate the contributions of various tissue components, such as the stroma or inflammatory infiltrates. A limiting factor for the broader application of mRNA analysis is that tumors, in the majority of cases, consist of a mixture of neoplastic and non-neoplastic cells. This issue is further compounded by the fact that many MIBCs induce a desmoplastic or inflammatory reaction [18].

Furthermore, significant heterogeneity exists within MIBC tumor proliferation, partly due to tumor progression phenomena and the multifocal nature of the neoplasm [36]. In transcriptomic studies, tumors with different cellular tumor types may cluster into the same group, whereas some tumors with identical cellular types may be grouped into different clusters. The most notable discrepancies were found between the increased mRNA expression of zinc finger E-box-binding homeobox 1 (ZEB1) and VIM in the basal category, whereas immunohistochemistry revealed that cells positive for VIM and ZEB1 were negative for basal markers (CK5/6 and CK14) and had a morphology more akin to mesenchymal rather than epithelial squamous cells [18].

Therefore, immunohistochemistry remains a reliable tool for identifying both intertumoral and intratumoral variability, as different neoplastic foci may coexist within the same tumor. In our study, we classified only the muscle-invasive component based on immunohistochemical profiles, not the superficial component. In many cases, we observed dissociation in both morphology and differentiation profiles between these components (data not shown).

A comprehensive analysis of histological variables, such as variants, LVI, PNI, tumor stage, and stromal TILs, confirmed the impact of TILs on overall and tumor-specific survival. The findings on tumor stage and LVI corroborate with several studies and a recent meta-analysis [32,35]. However, certain limitations in our study must be noted, including the lack of follow-up in 9.5% of patients, the retrospective nature of the study, the inclusion of specimens over 20 years, the absence of data on neoadjuvant or adjuvant chemotherapy and immunotherapy regimens, and the lack of information on disease stage in patients who died of non-tumor-related causes. Nonetheless, given the significant impact of stromal TILs on OS and TSS in both univariate and multivariate analyses, it would be valuable to reanalyze these cases using

standardized methods of lymphocytic infiltrate quantification, as has been done in other cancer types. Such an approach could provide a more accurate tumor stratification and improve our understanding of the impact of TILs on patient survival.

The present study demonstrated the feasibility of applying Lund University's molecular classification of MIBCs in routine diagnostic practice, offering valuable prognostic insights in a relatively wide population. Notably, all histological samples in this study were reviewed by two expert pathologists, ensuring consistency in tumor type classification between TURBT and cystectomy samples, which supports its clinical utility. Furthermore, tumor types significantly influenced survival outcomes, highlighting their relevance in patient stratification and the development of personalized treatment strategies. Future analyses integrating molecular classification with chemotherapy regimens are necessary to clarify the relationship between these factors.

This study also revealed distinct expression patterns of HER2/neu and p53 among tumor types, suggesting potential therapeutic targets. This observation highlights the need for further studies to explore targeted therapies for specific molecular subgroups, as reported by the DESTINY-PanTumor02 study in various solid tumors [37]. Moreover, the association between basal tumor types and higher stromal TILs suggests an immune-modulatory role, which could be exploited in immunotherapy strategies.

Among the limitations of this study, we acknowledge its retrospective nature, the absence of data on surgical and medical treatments received by the patients, and the lack of information on disease-free survival after cystectomy. Despite these limitations, our study provides compelling evidence supporting the integration of molecular classification into routine diagnostic workflows for MIBCs. This integration can enhance prognostic accuracy and guide therapeutic decisions. Further research is warranted to validate these findings in larger cohorts and to explore the clinical benefits of personalized treatment approaches based on molecular subtypes.

5. CONCLUSION

This study is the first to validate the use of TMA for attributing phenotype in large series with exceptional reliability and reproducibility compared to all histological sections. We also confirmed that TURBT can accurately predict the tumor type found in subsequent definitive surgical samples. Furthermore, this study demonstrated that TILs and MIBC tumor types have a significant, independent impact on overall and tumor-specific mortality, providing a foundation for improving the clinical management of patients with MIBC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was authorized by the Ethics Committee of the Emilia Ovest Area on 22/10/2019, protocol number 615/2019/TESS/AOUPR.

CONSENT FOR PUBLICATION

This is a retrospective study; thus, patient consent is not required for the publication of their data.

AVAILABILITY OF DATA

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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