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Triple-marker immunohistochemical assessment of muscle-invasive bladder cancer: Is there prognostic significance?

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Abstract

Background: Bladder cancer is the ninth most common cancer worldwide, and the third most common cancer in Lebanon. Immunohistochemistry (IHC) has been used to stratify muscle-invasive bladder cancer (MIBC) into different subtypes. However, to our knowledge, there exists no study that investigates the use of this low-cost technique to predict prognosis in bladder cancer patients in our region.

Aim: To examine the feasibility of low-cost triple-marker IHC assessment for MIBC subtyping in order to predict patients' survival and cisplatin sensitivity.

Methods and results: We collected the specimens of deceased patients diagnosed with MIBC on pathology at our institution. For each case, tumor tissue blocks were retrieved and stained for hematoxylin and eosin in addition to three molecular markers by IHC: cytokeratin 5/6, cytokeratin 14 staining basal BC, and GATA3 staining luminal BC. A cut-off of ≥20% was set as positive. Kaplan-Meier curves were built, factored by BC subtype, to predict overall survival (OS), disease-specific survival (DSS), and progression-free survival (PFS). Hazard ratios in Cox regression were also created accounting for oncological factors and BC subtype.

We categorized specimens as either luminal (GATA3 positive only) (n = 21; 56.7%) or as double-positive (GATA3 and basal cytokeratin 5/6 or cytokeratin 14 positive) (n = 16; 43.3%). The overall median survival was similar between the two categories (27.0 ± 4.82 months). Numbers favored luminal disease for PFS (Breslow P = .032). After adjusting for covariates, luminal molecular expression predicted PFS (0.28;

Abbreviations: CDDP, cisplatin; IHC, immunohistochemistry; MIBC, muscle-invasive bladder cancer; NAC, neoadjuvant chemotherapy; NMIBC, non-muscle-invasive bladder cancer; SCC, squamous cell carcinoma; TMA, tissue microarray analysis; TMT, trimodal therapy; TUBT, transurethral resection of bladder tumor; UC, urothelial carcinoma; UNC, University of North Carolina.

[0.09-0.94]). Yet, the Cox model was not able to identify any predictors of OS or DSS.

Conclusion: Specimens enriched with only a luminal molecular profile were more likely to exhibit cisplatin sensitivity. Despite the absence of guidelines recommending the utilization of molecular profiling in clinic practice, triple-marker IHC could serve as a potential low-cost prognostic indicator to identify patients at high risk of progression.

KEYWORDS

biomarkers, immunohistochemistry, Middle East, prognosis, urinary bladder neoplasms

1 | INTRODUCTION

Bladder cancer (BC) is the ninth most commonly diagnosed cancer worldwide with an estimate of 81 400 new cases and 17 980 deaths in the United States alone in 2020.[1] Bladder cancer rates are high in North America, Southern Europe, and certain nations of the Middle East and North Africa (MENA) region.[1] In the MENA region, Lebanon has the highest age-standardized incidence rate of BC making it the third most common cancer in Lebanon and second most common among Lebanese men.[2] According to the World Health Organization, this is attributed in big part to the regional record-breaking age-standardized prevalence of smoking (34% among individuals 15 years and older).[3]

Bladder cancer can either be non-muscle-invasive (NMIBC) or muscle-invasive (MIBC). In case of MIBC, radical cystectomy combined with perioperative cisplatin (CDDP)-based chemotherapy is the standard of care.[4,5] Additionally, the use of neoadjuvant chemotherapy (NAC) has a role in eliminating residual disease in the cystectomy specimen and prolonging patient survival.[6] The rise of genomic analysis has prompted classifying BC tumors according to their genetic aberrations and consequently identifying the appropriate treatment.[7] In 2014, the University of North Carolina (UNC) found that high-risk BC has a physiological development analogous to breast cancer and can be divided into either luminal or basal.[8] Since these two subtypes had distinct behaviors and chemotherapy sensitivities, the importance of subtype identification could impact treatment choice. Fortunately, immunohistochemistry (ICH) for markers such as GATA3 for luminal subtype and cytokeratin 5/6 for basal subtypes was 90% accurate at BC subtype identification.[9] In parallel, other forms of classification based on tissue microarray analysis (TMA) were later developed. Among the most prominent is the MD Anderson classification which divided MIBC into basal, luminal, and TP53-like and the Cancer Genome Atlas Project which divided it into four clusters.[10,11]

Genetic profiling is not only able to identify mutations but also offers targeted therapy.[5] Of interest is the TP53 gene mutation which was found to be responsible for CDDP resistance.[5,10] Nevertheless, RNA expression profiling requires complex protocols and is extremely expensive and challenging for clinical practice integration.[5] Nationwide in Lebanon, the health care sector is suffering from a shortage of medical supplies resulting in budget cutting.[12] Since BC rates are nationally on the rise, we aimed at examining the feasibility of a practical and low-cost triple-marker IHC assessment for MIBC subtyping in order to predict[1]: overall survival (OS),[2] disease-specific survival (DSS), and[3] progression-free survival (PFS) among patients who received CDDP therapy.

2 | METHODS

2.1 | Population selection

After receiving Institutional Review Board (IRB) approval, this pilot study comprised deceased patients only with a pathological diagnosis of MIBC at the American University of Beirut Medical Center who were selected over a span of 23 years (1993-2015). The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Charts were retrospectively analyzed using electronic health records (EHRs) for specimens collected from either transurethral resection of bladder tumor (TURBT) or cystectomy procedures. Only specimens with a pathologically confirmed muscle-invasive urothelial carcinoma (UC) of the bladder were selected from a total of 90 specimens. In congruence with previous studies, patients with NMIBC (n = 21) and non-UC pathology were excluded (n = 7).[10,13] Similarly, patients with no pathology on EHR (n = 13), duplicate pathology samples belonging to the same patient (n = 10), and patients lacking therapeutic history (n = 2) were omitted from the analysis, ending up with a total of 37 patients included.

2.2 | Immunohistochemistry

All specimens were collected at our institution, and standard tissue processing and block preparation were followed. Then, tissue blocks were stored at room temperature in secure areas.

For each case, four 3 μm sections were taken from each formalin-fixed paraffin-embedded tissue block. One of the sections was used for hematoxylin and eosin staining, and the other three sections were

used for IHC staining with each of cytokeratin 5/6, cytokeratin 14, and GATA3. While cytokeratin 5/6 and cytokeratin 14 are cytoskeletal constituents of the perinuclear cytoplasmic intermediate filament, GATA3 is a nuclear marker that detects the zinc-finger transcription factor protein encoded by GATA3 gene.[14,15] The basal markers included mouse monoclonal antibody against human cytokeratin 5/6 (D5/16B4 clone, prediluted and ready to use, Ventana Benchmark, Tucson, AZ) and mouse monoclonal antibody against human cytokeratin 14 (LL002 clone, prediluted and ready to use, BioGenex, Fremont, CA), whereas the luminal marker used was a mouse monoclonal antibody against GATA3 (L50-823 clone, prediluted and ready to use, Ventana Benchmark, Tucson, AZ). These markers are sensitive enough to categorize MIBC according to UNC groups.[9] Then, the antibody reactivity was assessed using Ventana Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ) for GATA3 and Bond Polymer Refine Detection (Leica Biosystems, Newcastle, UK) for cytokeratin 5/6 and cytokeratin 14 according to the manufacturers' instructions.[16] Adequate positive and negative internal controls assured the validity of stain interpretation. In specific, adequately stained positive controls confirmed tissue antigenicity, tissue validity, and stain reliability.

A specialized pathologist at our institution examined the stained slides using a light microscope. Each immunohistochemistry (IHC) slide (3 μm sections) was granted a percentage and intensity of expression of tumor cells. Since cellular expression is not uniform throughout a single given tumor, for each stain, a staining percentage of <20% was considered as negative expression and a staining percentage ≥ 20% as positive expression.[9] The percentage of positive cells was estimated in whole section by comparing the number of stained tumor cells to the total number of tumor cells present on the hematoxylin and eosin slide. Extracellular staining and edge effect were disregarded. Then, specimens were categorized as either luminal (GATA3 positive only) or double-positive (GATA3 and basal cytokeratin 5/6 or cytokeratin 14 positive). Figure 1 exhibits an example of luminal and an example of double-positive IHC results.

2.3 | Variable selection

Retrospective data for patients' demographics, comorbidities, stage of disease, presence of metastasis, and type of surgical intervention (TURBT or cystectomy) were collected. In the case where the same patient had pathological specimens from a TURBT and a cystectomy, the latter was chosen due to better disease sampling. Additionally, therapeutic interventions including intravesical Bacillus Calmette-Guerin/chemotherapy, systemic chemotherapy, and radiation were recorded. The primary endpoint was disease-specific survival (DSS), which was calculated from date of surgery until death censoring the date of last contact for living patients. Moreover, BC-related death was defined as documented death resulting from one of the following: severe progressive disease, organ failure, urosepsis, or thromboembolic events. Furthermore, progression-free survival (PFS) was defined as the arithmetic difference between the date of chemotherapy and the date of confirmed disease progression censoring the date of last recorded imaging demonstrating no disease progression. Progression of disease was evidenced by computerized tomography scans evaluated by radiologists at our institution to assess burden of disease.

2.4 | Statistics

The two groups (luminal and double-positive) were compared using Mann-Whitney *U* test for continuous variables and chi-squared test for categorical variables. Fisher's exact test was used for categorical variables when conditions for chi-squared test were not satisfied. Kaplan-Meier curves were constructed for OS, DSS, and PFS. The curves were also factored by BC molecular expression. The Breslow (Generalized Wilcoxon) and the log-rank test (Mantel-Cox) were used to detect significance between the two groups. Then, hazard ratios estimated in Cox regression based on DSS and PFS were reported with their corresponding 95% confidence intervals (CI). The model for DSS was conducted on the total number of patients, while the model perioperative chemotherapy response was run only among those who either received CDDP. The models were adjusted for age, comorbidities, whether cystectomy was done, stage of the disease, and UC histological profile. The stage of disease was denoted for each patient using the combination of clinical, pathological, and radiological findings. All analyses were conducted on the Statistical Package for the Social Sciences IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, NY), and a $P < .05$ was set as a cut-off for significance.

3 | RESULTS

The cohort had an average age of 72.1 ± 10.8 and was mostly constituted of males (86.5%) (Table 1). The proportion of patients with hypertension, diabetes, and cardiovascular disease were 29.7%, 18.9%, and 40.5%, respectively. At diagnosis, 51.9% of the patients had a stage ≥ III BC. Among the specimens examined, 35.1% of them were taken from cystectomy pathologies. Furthermore, although most patients in the cohort received adjuvant CDDP (75.5%), 83.8% of the patients eventually developed metastasis. Metastasis mostly occurred in the lymph nodes (n = 25; 67.6%), the lungs (n = 18; 48.6%), and the bones (n = 16; 43.2%).

We categorized specimens as either luminal (GATA3 positive only) (n = 21; 56.7%) or as double-positive expressing both luminal GATA3 and either basal cytokeratin 5/6 or cytokeratin 14 (n = 16; 43.3%) (Figures 1 & 2). Moreover, six specimens (16.2%) were triple-marker positive, and none of the specimens stained solely for basal markers. One specimen exhibited glandular differentiation and another specimen displayed squamous differentiation. Although both of these specimens were double-positive, no significant morphological difference was apparent between the two groups. Furthermore, both groups were comparable in age, comorbidities, smoking status, cancer stage, and cystectomy rates (Table 1). Similarly, the difference in the

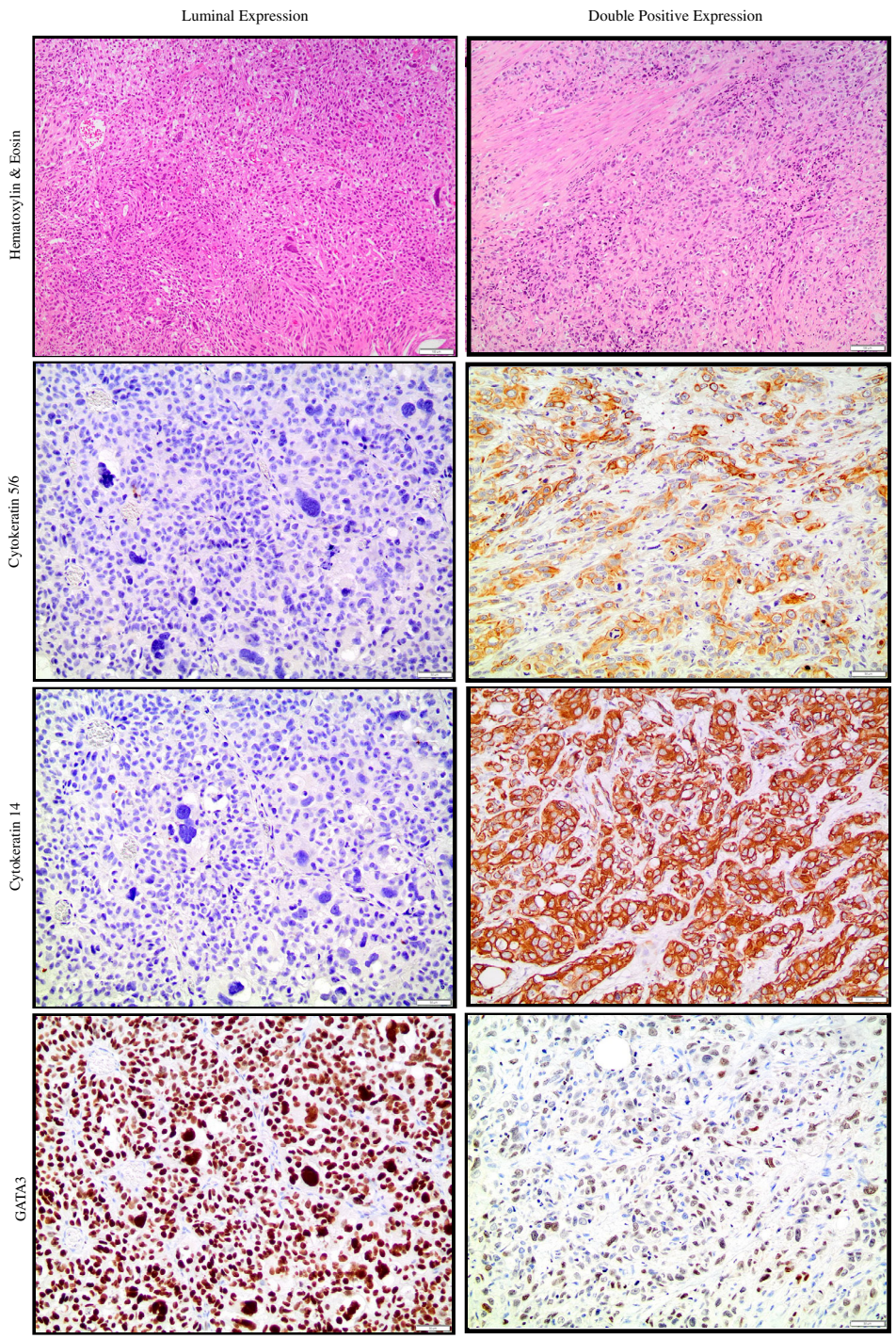

FIGURE 1 Characteristic expression of luminal or double-positive with cytokeratin5/6, cytokeratin 14, and GATA3 (in color online only). Hematoxylin and eosin were examined at ×10 magnification. The molecular expression stains were examined at ×20 magnification

rate of radiation, intravesical therapy, neoadjuvant, and systemic chemotherapy or trimodal therapy was not significant.

This cohort had a median survival of 27.0 ± 4.82 months. The median overall survival (OS) was similar between the two groups (P = .393) (Figure 3). For disease-specific survival (DSS), although nonsignificant, numbers favored luminal disease in comparison to double-positive disease (25.0 ± 7.43 months vs 19.0 ± 7.19 months; P = .181). Luminal disease also showed an early statistically superior progression-free survival (PFS) illustrated by a significant Breslow (Mantel-Cox test) P = .032. Ultimately, the PFS for luminal and double-positive molecular expression converges at 28 months resulting in a nonsignificant log-rank test (P = .11).

After adjusting for patients' age, comorbidities, and the therapy received, the Cox model was not able to identify any predictors of OS (data not shown) or DSS (Table 2). Among those who received perioperative CDDP chemotherapy, patients with luminal disease were more likely to have a longer progression-free time interval with odds ratio of (OR = 0.28; 95%CI 0.09-0.94) (Table 2). Effectively, tumor molecular expression was the sole predictor of progression-free survival, where luminal disease was associated with a better prognosis.

TABLE 1 Patient characteristics, comorbidities, and oncological profile of patients with basal and double-positive molecular expression in muscle-invasive bladder cancer

Variable	Luminal (N = 21) $\bar{x} \pm SD$; n (%)	Double-Positive (N = 16) $\bar{x} \pm SD$; n (%)	*P* value
Age	70.38 ± 10.37	74.31 ± 11.37	.238
Male	18 (85.7%)	14 (87.5%)	1
Cystectomy	8 (38.1%)	5 (35.7%)	.886
Intravesical treatment	7 (41.2%)	6 (46.2%)	.785
Chemotherapy	18 (90.0%)	9 (64.3%)	.097
Bladder cancer-related death	17 (94.4%)	12 (92.3%)	1
Adjuvant chemotherapy	11 (73.3%)	2 (25.0%)	.109
Neoadjuvant chemotherapy	3 (14.3%)	2 (12.5%)	1
Radiation	9 (50.0%)	3 (25.0%)	.260
TMT	3 (18.8%)	2 (18.2%)	1
Metastasis	19 (90.5%)	12 (75.0%)	.371
Stage ≥ III	9 (56.3%)	5 (45.5%)	.581
Hypertension	8 (38.1%)	3 (18.8%)	.285
Diabetes	2 (9.5%)	5 (31.3%)	.202
Cardiovascular	7 (33.3%)	8 (50.0%)	.306
Smoker	9 (64.3%)	7 (70.0%)	1
Other primary cancers	2 (9.5%)	1 (6.3%)	1

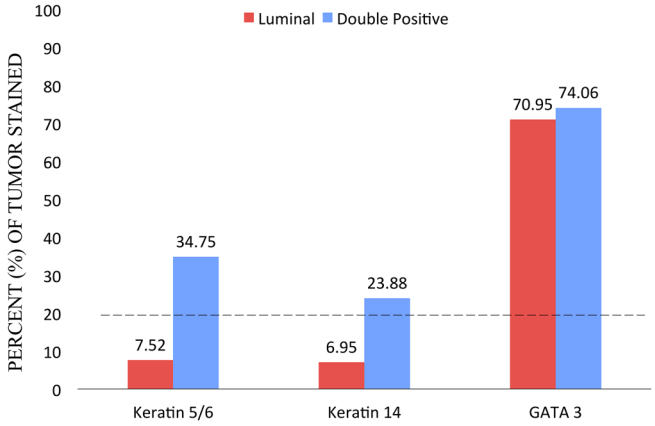

FIGURE 2 Percent of tumor stained with GATA 3, keratin 5/6, and keratin 14, respectively, in luminal and double-positive muscle-invasive bladder cancer (in color online only). Line at 20% represents the cut-off used to label a bladder cancer as either luminal or double-positive

4 | DISCUSSION

Using IHC staining for light microscopy, we classified MIBC specimens into two categories: luminal and double-positive disease, where the former expressed GATA3 alone and the latter coexpressed both luminal (GATA3) and basal markers (cytokeratin 5/6 and/or 14). In our series, 16.2% of the specimens were triple-marker positive, and none of the specimen expressed basal markers solely. The coexpression of both markers had an unfavorable effect on progression-free survival. Moreover, although luminal disease was associated with a superior disease-specific survival, molecular subset of BC was not a predictor in the Cox regression model probably due to small sample size.

The results of our cohort reflect the findings of other studies which demonstrate that a basal molecular expression compromises OS and DSS.[10,17] Effectively, basal MIBC expresses cytokeratin 5/6 and 14, which are cancer stem-cell biomarkers typical of epithelial-mesenchymal transition, granting the disease its aggressive biology.[10,17] In our series, the basal subtype is anticipated to correspond to the double-positive group. This is also evident clinically where patients with basal subtype BC often present with advanced stage of the disease.[10,17] In parallel, a similar behavior was previously reported in malignant breast lesions.[18]

Moreover, our study determined that a luminal MIBC was associated with increased odds of PFS in comparison to double-positive disease. In contrast, several studies affirmed that patients with basal disease had improved OS from CDDP based neoadjuvant treatment in comparison to tumors with either p53-like or luminal molecular signatures.[5,19] Nevertheless, most CDDP resistance could be explained by the accumulation of TP53 mutations.[5,7,10] Although the MD Anderson group divided BC into luminal, basal, and TP53-like, the latter group is a heterogeneous mixture of basal and luminal precursors which have a genetic aberration of the cell cycle tumor suppressor.[10] The meta-analysis carried by Dadhania et al in 2016 revealed that prognosis worsened in the following order: double-negative, non-p53 luminal, p53-like luminal, non-p53 basal, and p53-like basal.[9]

Since our project relied on the attempt to identify the clinical implication of University of North Carolina (UNC) categorization, p53 expression was not carried out. Yet, a meta-cohort analysis of 2411 urothelial bladder tumors recently divided bladder cancer into six molecular subtypes.[20] While the HER2-like, papillary-like, and

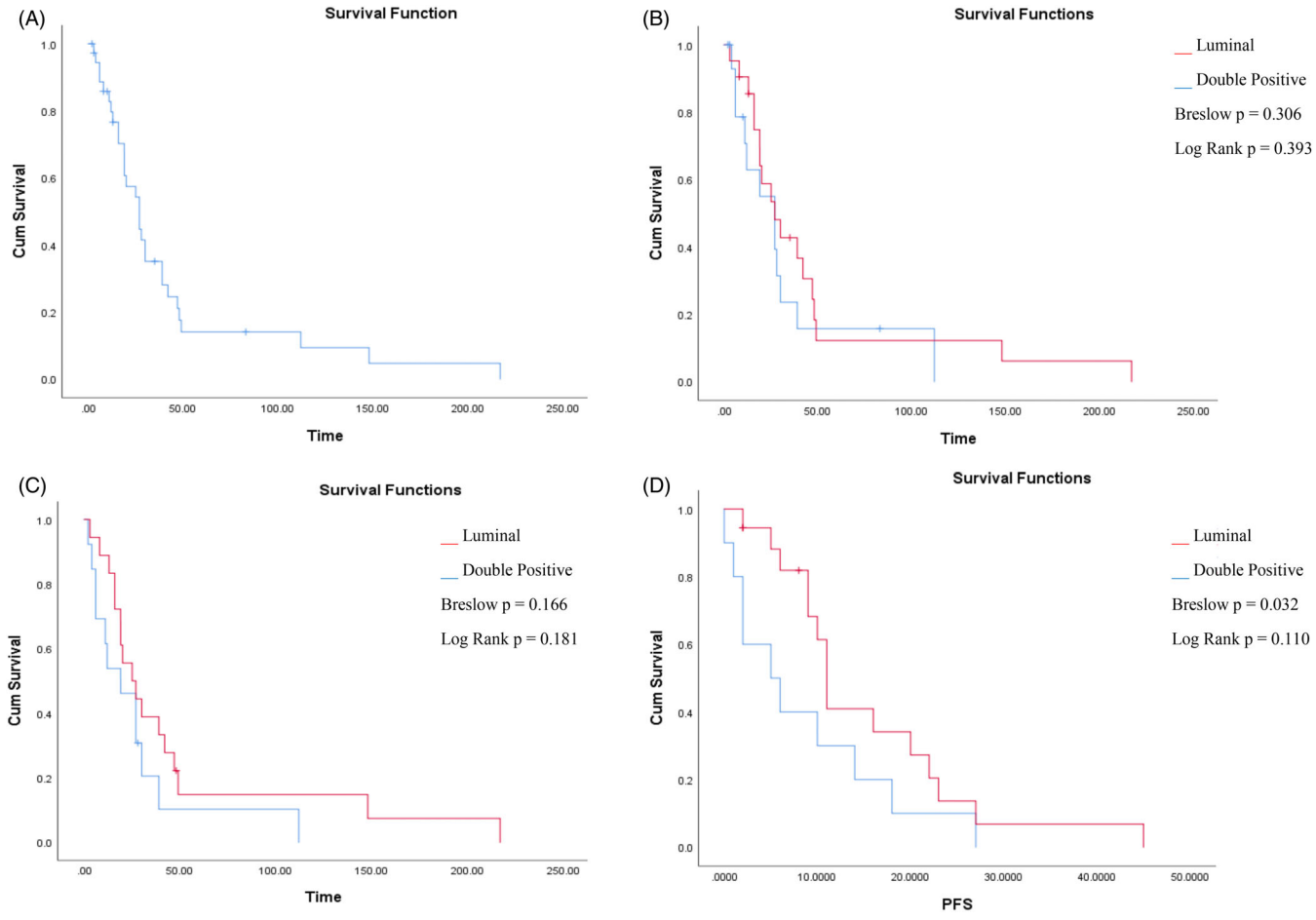

FIGURE 3 Kaplan-Meier Curves (in color online only)

TABLE 2 Hazard ratio estimated in Cox regression based on disease-specific mortality and progression-free survival in patients with muscle-invasive bladder cancer

	Disease-specific Mortality (N = 33)		Progression-free Survival (N = 28)	
Variable	HR (95% CI)	*P* value	HR (95%CI)	*P* value
Age	1.07 (0.61-1.89)	.805	0.91 (0.57-1.44)	.908
Hypertension	0.21 (0.03-1.45)	.113	0.51 (0.12-2.23)	.371
Diabetes	0.25 (0.04-1.70)	.246	0.40 (0.06-2.69)	.348
Cardiovascular disease	2.44 (0.46-12.97)	.122	0.894 (0.30-2.65)	.840
Cystectomy	0.64 (0.12-3.26)	.586	0.73 (0.17-3.01)	.661
Metastasis	1.57 (0.11-1.53)	.145	1.70 (0.20-14.78)	.270
Luminal expression[a]	4.36 (0.96-19.91)	.057	0.28 (0.09-0.94)	.039
Chemotherapy	1.69 (0.32-8.93)	.540	N/A	N/A
Radiation	2.94 (0.40-21.79)	.292	N/A	N/A
Intravesical BCG or chemotherapy	0.42 (0.12-1.53)	.188	N/A	N/A

Abbreviations: 95% CI, 95% confidence interval; HR, hazard ration; N/A, not applicable.
[a]Luminal IHC expression takes double-positive IHC as a reference.

luminal-like captured the luminal UNC category, the squamous-cell-carcinoma-like (SCC), neural, and mesenchymal were subtypes of the basal UNC category.[20] The authors found that the SCC and the HER2-like specimens were enriched with TP53 mutations.[20] Furthermore, SCC proved to have the worse prognosis.[20] Since TP53 inactivation is found in the majority of MIBC, our findings suggest that

double-positive disease could be a surrogate marker of more aggressive biology.[21] Nonetheless, further subtyping of the UNC luminal category in future studies can prove to be beneficial; especially that the luminal-like and papillary-like subtypes are characterized by 9q deletions analogous to the ones found in NMIBC.[5,20] Thirteen of our 37 patients presented originally with NMIBC and progressed to MIBC while receiving intravesical therapy. It is proposed that FGFR3 mutations in NMIBC might be responsible for disease stability.[22] However, we did not stain our specimens for FGFR3, and so we cannot certainly presume that these patients lack FGFR3 mutations which might explain why they progressed.

GATA3 transcription factor expression is expected when examining urothelial tissue because it is responsible for its differentiation and inhibition of bladder cancer development and progression. Li et al demonstrated that the down-regulation of GATA3 expression was correlated with high-grade invasive bladder tumors, as this phenomenon promoted neoplastic transformation, migration, and invasion of urothelial-derived tumor cells.[23] In our study, the groups double-negative and basal were not present. Wang et al faced the same difficulty when trying to identify the basal subtype as they noticed that a strong cytokeratin 5/6 expression was not necessarily correlated with a negative GATA3.[13] Moreover, the authors reported that 48.3% of specimens (vs 43.2% in our series) coexpressed basal and luminal markers.[13] Similar to our results, the authors found that diffuse (>85% positivity) GATA3 expression was protective and was associated with 0.27 (vs 0.28 in the luminal category of our cohort) the odds for recurrence-free survival.[13]

4.1 | Limitations

Through this paper, we assessed OS, DSS, and PFS among molecular subtypes of MIBC using IHC for light microscopy. The cohort at hand neither exhibited double-negative nor pure basal histology. This could be attributed to either ethnic differences or due to the nature of our cohort, as all specimens were procured from patients with various stages of disease.[13] In addition, all tissues were examined by light microscopy, which could bring about systematic human error as it is an operator-dependent modality. Nevertheless, the same pathologist read all the microscopy slides thereby decreasing interobserver variation. Furthermore, reverse-phase protein arrays and IHC readings using TMA would have improved the accuracy of our results, but, these modalities are costly, time-consuming, and require technical expertise.[5] Yet the adherence to IHC protocols and the pathologist's expertise in the field guarantee our study's internal validity.

We also postulate that the relatively small number of the cohort explains the lack of significance for OS and DSS. Another limitation to our study is the use of specimens from both TURBT and radical cystectomy as tumor sampling could yield different results. Besides, tumor profiling could fluctuate not only in location, due plasticity according to cancer cells' microenvironment, but also in time as cancer cells acquire further mutations.[5] Other studies also illustrated the discordance of IHC molecular subtyping between bladder tumors and their synchronous lymph node metastases.[24] Bladder cancer does not possess a homogenous molecular expression. In fact, intratumor heterogeneity renders bladder cancer subtyping and treatment difficult.[25] In our study, tissue obtained from TURBT might explain the lack of significance of other covariates or outcomes. Therefore, future works should perform multiple sectioning of tumor blocks to eliminate the possibility of intratumor molecular heterogeneity.[25]

5 | CONCLUSION

Although no recommendations can be issued regarding the clinical role of triple-marker IHC assessment for predicting prognosis in MIBC, results from our study pave the way for future studies to elucidate the roles of IHC biomarkers such as cytokeratin 5/6, cytokeratin 14, and GATA3 in identifying patients at high risk of progression. Herein, we found that specimens enriched with only a luminal molecular profile exhibited a better prognosis. Thus, future prospective studies with larger number of patients are needed to confirm our findings.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interests other than the funding source stated above.

AUTHOR CONTRIBUTIONS

M.L.; Formal analysis, visualization, writing-original draft. J.N.; Data curation, project administration, writing-original draft. D.M.; Conceptualization, funding acquisition, supervision, writing-review and editing. W.A.K.; Funding acquisition, methodology, supervision, writing-review and editing. A.T.; Conceptualization, investigation, methodology, project administration, supervision, writing-review and editing. A.E.H.; Conceptualization, data curation, funding acquisition, methodology, resources, supervision, writing-review and editing.

ETHICS APPROVAL AND PATIENT CONSENT

The Institutial Review Board at the American University of Beirut has reviewed the proposal on September 25th, 2017, and declared that the research does not consititute “Human Research” as defined by DHHS and USFDA guidelines due to the nature of the study (post-mortem samples).

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

DATA AVAILABILITY STATEMENT

The data is the property of the American University of Beirut Medical Center and prior approval is necessary to share the data. The data is available upon request through the principal investigator.

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