Role of Ki-67, MRE11, and PD-L1 as Predictive Biomarkers for Recurrence Pattern in Muscle-invasive Bladder Cancer

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Abstract. *Background/Aim:* Muscle invasive bladder cancer (MIBC) is an aggressive disease with high rates of local recurrence following radical cystectomy (RC). Currently, there are no clinically validated biomarkers to predict local only recurrence (LOR) and guide adjuvant treatment decisions. This pilot study evaluated the role of Ki-67, MRE11 and PD-L1 as predictive biomarkers for recurrence patterns in patients undergoing RC for MIBC. *Patients and Methods:* Our institutional cystectomy database containing cases from 1992-2014 was queried for patients with local only recurrence (LOR), and case-matched to patients with distant recurrence (DR) and no recurrence (NR). Clinicopathological data were collected and a tissue microarray was analyzed for presence of Ki-67, MRE11, and PD-L1 using immunofluorescence and immunohistochemistry. *Results:* Pathologic specimens from 42 patients (18 NR, 16 LOR, and 8 DR) were reviewed. Compared to normal bladder tissue, tumors had increased expression of Ki-67 ($p<0.01$) and PD-L1 ($p<0.05$). High Ki-67 was associated with recurrence pattern (local vs. distant) on univariate analysis ($p<0.05$). Ki-67 cell density varied by

recurrence type: LOR (1354 cells/mm²), DR (557 cells/mm²) and NR (1111 cells/mm²) ($p=0.034$). *Conclusion:* Our selected biomarkers could distinguish MIBC from normal bladder tissue but could not classify samples by recurrence pattern.

Muscle-invasive bladder cancer (MIBC) carries a relatively poor prognosis due to high rates of local and distant recurrence following radical cystectomy (1-2). Although it confers an overall survival advantage, neoadjuvant chemotherapy (NAC) does not decrease rates of pelvic recurrence, which can occur in 8-40% of patients with locally advanced disease (3-6). Post-operative radiation (PORT) can reduce pelvic recurrence rates, but appropriate patient selection remains controversial. Decisions regarding PORT are currently based on clinicopathologic features at the time of cystectomy.

Molecular subtypes of MIBC have been shown to better stratify outcomes compared to clinicopathologic features alone (7-10). Prior studies have utilized whole transcriptome sequencing to classify tumors into basal, luminal, and high TP53-expressing molecular subtypes, which are useful in predicting the overall risk of recurrence (local and distant) and responsiveness to chemotherapy (11-13). Specific biomarkers including MRE11, PD-L1 and Ki-67 have been shown to be prognostic and, in some cases, predictive of oncologic outcomes following definitive management of MIBC (10, 14-16). However, there are no clinically validated biomarkers to predict local recurrence and guide treatment decisions including use of PORT.

In this pilot study, we evaluated MRE11, PD-L1, and Ki-67 expression in patient tumors and normal bladder tissue using quantitative immunohistochemistry (IHC) and immunofluo-

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rescence (IF) assays and compared our findings to oncologic outcomes in our institutional cystectomy database. Our primary objective was to define a molecular signature for local recurrence to improve selection of patients for PORT.

Patients and Methods

Cystectomy database. Following IRB approval (IRB#: HS-01B014-CR015), our institutional bladder cancer database was queried for patients that experienced a local only recurrence (LOR) following cystectomy from January 1992 through December 2014. LOR was defined as radiographically detected pelvic recurrence up to the level of the common iliac vessels with no disease detected outside of the pelvis. The database was then queried to case-match the 20 LOR patients to 20 patients without recurrence (NR) and 20 patients experiencing a distant recurrence (DR), which was defined as an extra-pelvic first recurrence. The variables used to match patients were stage, type of urinary diversion, use of NAC, and the decade the operation was performed.

Pathologic review. Specimens from 42 patients (18 NR, 16 LOR, and 8 DR) had sufficient tissue for review. A single pathologist examined all specimens to identify appropriate areas for construction of a tissue microarray (TMA). The TMA was built from formalin-fixed, paraffin embedded (FFPE) tissue and probed using antibodies directed against MRE11 (Abcam, EPR3471), PD-L1 (CST, E1L3N), and Ki-67 (Abcam, polyclonal). IF was performed for all three proteins (Figure 1) and IHC was performed for PD-L1 and Ki-67. Normal bladder tissue adjacent to each tumor (n=42) from RC specimens was also included in the TMA.

Multiplex immune panel procedure. FFPE tissue samples were immunostained using the PerkinElmer OPAL TM 7-Color Automation IHC kit (Waltham, MA, USA) on the BOND RX autostainer (Leica Biosystems, Vista, CA, USA). DAPI counterstain was applied to the multiplexed slide and was removed from BOND RX for coverslipping. Autofluorescence slides (negative control) were included, which used primary and secondary antibodies omitting the OPAL fluors and DAPI. All slides were imaged with the Vectra®3 Automated Quantitative Pathology Imaging System (Akoya Biosciences, Marlborough, MA, USA).

Quantitative image analysis. Multi-layer TIFF images were exported from inForm (PerkinElmer) and loaded into HALO (Indica Labs, Albuquerque, NM, USA) for quantitative image analysis. The tissue was segmented into individual cells using the DAPI marker which stains cell nuclei. A positivity fluorescent threshold was determined for each biomarker based on published staining patterns and intensity for that specific antibody (17-19). After setting a positive threshold for each staining biomarker, the entire image set was analyzed with the created algorithm.

Statistics and model building. Four IHC measurements (2 per biomarker) and nine IF measurements (3 per biomarker) were recorded for a total of 13 measurements for each TMA core. IHC measurements included H score and Combined Positive Score (CPS) (<10, >10) for PD-L1 and H score and percentage (<1, >1) for Ki-67. IF variables included absolute positive cells per TMA core, percent positive cells (number of positive cells / total number of cells

Table I. Patient characteristics.

	n (%) (N=42)
Age, years	70 (53-85)*
Gender	
Male	26 (62%)
Female	16 (38%)
pT stage	
pT1	1 (2%)
pT2	7 (17%)
pT3	22 (52%)
pT4	12 (29%)
pN stage	
pN0	15 (36%)
pN1	10 (24%)
pN2	14 (33%)
pN3	3 (7%)
Margin Status	
Positive	6 (14%)
Negative	35 (83%)
Unknown	1 (2%)
Presence of LVSI	
Yes	22 (52%)
No	17 (40%)
Unknown	3 (7%)
Neoadjuvant chemo	
Yes	10 (24%)
No	32 (76%)

*Data shown as median (range). LVSI: Lymphovascular space invasion.

per core ×100), and positive cell density (cells/mm²) for each biomarker. Percentage and density of cells expressing the biomarkers were included to normalize the data and account for varying number of total cells in each core. *t*-Tests, univariate and multivariate logistic regressions, and Cox proportional hazard models were performed to assess the association of variables with oncologic outcomes. Principal component analyses (PCAs) were performed to separate patients by recurrence pattern using the “prcomp” function provided by R statistical software. Multidimensional scaling maps were constructed.

Results

Patient characteristics. Patient characteristics are shown in Table I. The median follow-up was 12.2 years and the median age was 70 years (range=53-85). Sixteen patients were female (38%). A majority of patients had locoregionally advanced disease with 32 (81%) having T3/T4 disease and 27 (64%) with positive lymph nodes. Six patients (14%) had positive margins and 22 (52%) had lymphovascular space invasion (LVI). Ten patients (24%) received NAC. None received radiation.

Tumor vs. normal tissue. There were notable differences between tumor and normal tissue with regard to biomarker

Figure 1. Immunofluorescence of normal tissue (a) and tumor (b) for MRE11 (green), PD-L1 (yellow), and Ki-67 (red) using Vectra 3 scans at 20 $\times$ magnification. Blue represents DAPI nuclear stain. Note: scale bar represents 50 micrometers.

expression (Table II, Figure 1). On IHC, tumor cells had an increased Ki-67 H score (33.3 vs. 0, $p < 0.001$) and higher PD-L1 H score (6.93 vs. 0, $p = 0.034$) and on IF, tumor had increased PD-L1 positive cells (237 vs. 35, $p = 0.019$) and PD-L1 positive cell density (274 vs. 53.4 cells/mm², $p = 0.034$) compared to normal tissue. There were no differences in MRE11 expression between tumor and normal tissue.

Associations with the pattern of recurrence and survival. On student's *t*-test (univariate analysis), three Ki-67 IF measurements, Ki-67 positive cells, percent Ki-67 positive cells, and Ki-67 positive cell density, were associated with a higher likelihood of LOR versus DR ($p = 0.036$, $p = 0.026$, and $p = 0.034$, respectively; Table III). Ki-67 cell density varied by recurrence type: LOR (1,354 cells/mm²), DR (557 cells/mm²) and NR

Table II. Tumor vs. normal tissue values for Ki-67, MRE11, and PD-L1 biomarkers with comparative two-tailed *t*-tests.

Molecular variable	Mean for normal	Mean for tumor	Tumor compared to normal	<i>t</i> -Test <i>p</i> -Value
PDL1 H score IHC	0	6.93	Increased	0.034
PDL1 CPS IHC (#<10, #>10)	(3, 0)	(40, 2)	No difference	1.00
Ki67 H score IHC	0	33.3	Increased	0.00016
Ki67 percentage IHC (<1, >1)	(3, 0)	(33, 9)	No difference	0.88
MRE11 positive cells per 20× field	912	2319	Increased	0.060
% MRE11 positive cells	66.2	52.8	Decreased	0.21
MRE11 positive cell density (cells/mm ²)	1,355	2,749	Increased	0.17
Ki67 positive cells per 20× field	764	923	Increased	0.84
% Ki67 positive cells	39.0	22.2	Decreased	0.60
Ki67 positive cell density (cells/mm ²)	1,183	1,115	Decreased	0.96
PD-L1 positive cells per 20× field	35	237	Increased	0.019
% PD-L1 positive cells	2.00	4.98	Increased	0.12
PD-L1 positive cell density (cells/mm ²)	53.4	274	Increased	0.034

IHC: Immunohistochemistry; CPS: combined positivity score. Significant *p*-values (*p*<0.05) are shown in bold.

Table III. Two-tailed *t*-tests for recurrence patterns based on Ki-67, MRE11, and PD-L1 expression.

	Mean for NR	Mean for LOR	Mean for DR	<i>t</i> -Test <i>p</i> -Values		
				NR vs. LOR	NR vs. DR	LOR vs. DR
PDL1 H score IHC	1.22	12.5	8.63	0.14	0.39	0.72
PDL1 CPS IHC (#<10, #>10)	(17, 1)	(13, 3)	(7, 1)	0.51	1.00	1.00
Ki67 H score IHC	30.7	43.9	17.9	0.57	0.30	0.19
Ki67 percentage IHC (#<1, #>1)	(14, 4)	(12, 4)	(7, 1)	1.00	0.97	0.86
MRE11 Positive Cells per 20× field	2,301	2,394	2,193	0.99	0.79	0.65
% MRE11 Positive Cells	51.3	55.1	51.2	0.80	0.99	0.70
MRE11 Positive Cell density (cells/mm ²)	2,676	2,926	2,530	0.77	0.77	0.44
Ki67 Positive Cells per 20× field	946	1,084	489	0.74	0.069	0.036
% Ki67 Positive Cells	21.1	28.2	11.0	0.42	0.088	0.026
Ki67 Positive Cell density (cells/mm ²)	1,111	1,354	577	0.59	0.082	0.034
PD-L1 Positive Cells per 20× field	156	345	202	0.34	0.82	0.58
% PD-L1 Positive Cells	3.10	7.11	4.92	0.28	0.72	0.71
PD-L1 Positive Cell density (cells/mm ²)	180	397	231	0.34	0.83	0.58

NR: No recurrence; LOR: local only recurrence; DR: distant recurrence; IHC: immunohistochemistry; CPS: combined positivity score.

(1,111 cells/mm²). On multivariate Cox proportional hazards model analysis, older age (HR=1.08, *p*=0.015), local recurrence (HR=4.14, *p*=0.022), distant recurrence (HR=50.7, *p*<0.001), and positive LVI (HR=5.96, *p*=0.015) were associated with worse overall survival. The following were associated with lower recurrence free survival (RFS): age (HR=1.09, *p*=0.016), pT3 (HR=8.16, *p*<0.001), pT4 (HR=1.64, *p*=0.004), pN2 (HR=4.60, *p*=0.009), pN3 (HR=7.64, *p*=0.010), and LVI (HR=41.5, *p*<0.001). The only biomarker associated with improved OS was PD-L1 positive cells (HR=1.03, *p*=0.036), although it was not associated with RFS.

Developing a molecular signature. Of the 42 pathologic specimens included in this study, one sample was excluded

due to unknown margin status bringing the total number of cases included in the PCAs to 41. We performed a series of PCAs to separate patients by recurrence pattern. The first analysis included all 15 biomarker variables (Figure 2), the second included all 22 clinical and biomarker variables, and the third was limited to margin status and Ki-67 cell density which were significant on UVA. None of the PCAs were successful in distinguishing samples by recurrence pattern.

Discussion

Local recurrences occur in up to 40% of patients following definitive management of MIBC and are a significant source of morbidity and mortality (4-6). This pilot study evaluated

Figure 2. Multidimensional scaling map depicting a principal component analysis (PCA) of all 15 biomarker variables. None of the PCAs used in this study was effective in separating patient samples by recurrence patterns. LOR: Local only recurrence; DR: distant recurrence; NR: no recurrence.

the ability Ki-67, MRE11, and PD-L1 to predict recurrence pattern and inform adjuvant treatment decisions.

Several findings in the current study are worth highlighting. First, we found significant differences in biomarker expression, specifically Ki-67 and PD-L1, which was higher in MIBC tumors compared to surrounding bladder tissue (Table II, Figure 1). These findings are consistent with known hallmarks of cancer including increased cellular proliferation and evasion of immune system detection. Regarding overexpression of PD-L1, this underscores the value of immune checkpoint inhibitor therapy as a treatment modality for bladder cancer. Second, our data show that high Ki-67 cell density may be associated with increased rates of local versus distant recurrence (Table III). Prior work has shown that a

high Ki-67 is associated with aggressive disease, recurrence, and cancer-specific survival following cystectomy but not specifically local recurrence (14-16, 20). Our data also suggest that tumors with high Ki-67, while associated with higher rates of proliferation, may not necessarily have a tendency to metastasize. Finally, although MRE11 has been previously established as a predictive biomarker in the definitive management of MIBC (14, 21-22), there were no notable findings in the current study regarding MRE11.

This study may lack the statistical power needed to detect a true difference in recurrence patterns based on biomarker expression due to small sample size. Another potential limitation is reliability of IHC and IF techniques which are only semi-quantitative and dependent on many difficult to control

variables including antibody choice and concentration, fixation technique, and inconsistency in specimen handling, technique, and interpretation. To minimize these errors, we used a highly standardized protocol and automated scoring systems for both IHC and IF. Lastly, the ability to predict recurrence pattern following cystectomy may require more than 3 carefully selected biomarkers. Recent studies have shown that in addition to DNA damage response, proliferative, and PD-1/PDL1 axis biomarkers, molecular subtypes, microRNAs, circulating tumor DNA, and tumor hypoxia modification biomarkers may all be contributing revealing an ever increasingly complex landscape with additional research opportunities (23).

In conclusion, two biomarkers used in this study, Ki-67 and PD-L1 were useful in distinguishing tumor from normal tissue, and high Ki-67 may be associated with LOR. Further work is needed to develop a molecular signature predictive of local recurrence in MIBC following radical cystectomy to guide adjuvant treatment decisions.

Conflicts of Interest

Dr. Magliocco reports personal fees from Protean BioDiagnostics Inc, outside the submitted work. Dr. Mouw reports grants from Pfizer, personal fees from UpToDate, and personal fees from OncLive, outside the submitted work. Drs. Fossum, Xiong, Daneshmand, Aron, Manojlovic, McCarthy, Phuong, Dorff, Bhanavdia and Ballas have no conflicts of interest to report. All authors agree with being listed as an author. The work has not been published before or being considered for publication elsewhere. There were no human or animal subjects.

Authors' Contributions

KB, AM, and SD contributed to the conception of the study. YX, MA, ZM, SM and CP performed the study. CCF, LKB, YX and AM contributed to the interpretation and analysis of data. The article was written by CCF, LKB, AM, SD, KWM, TD and SKB. All authors had access to the data.

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