Role of Ki-67, MRE11, and PD-L1 As Predictive Biomarkers for Recurrence Pattern in Muscle Invasive Bladder Cancer Following Radical Cystectomy

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Abstract

Background

Muscle invasive bladder (MIBC) cancer 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.

Methods:

Our institutional cystectomy database containing cases of urothelial MIBC from 1992–2014 was queried for patients with LOR and case-matched with patients who had distant recurrence (DR) and no recurrence (NR). Clinical and histopathological data were collected from selected patients and a tissue microarray was built and analyzed for presence of Ki-67, MRE11 and PD-L1 using immunofluorescence (IF) and immunohistochemistry (IHC). Univariate, multivariate, and principal component analyses (PCA) were performed to evaluate association of these biomarkers with recurrence pattern.

Results:

Pathologic specimens from 42 patients (18 NR, 16 LOR and 8 DR) were reviewed. Compared to adjacent normal bladder tissue, tumors had increased expression of PD-L1 on both IHC and IF ($p < 0.05$) and higher Ki-67 H-score on IHC (33 vs 0, $p < 0.01$). A high Ki-67 was associated with recurrence pattern (local vs distant) on univariate analysis ($p < 0.05$) and on multivariate regression, having positive surgical margins was associated with local recurrence ($p = 0.04$). Ki-67 cell density varied by recurrence type: LR (1354 cells/mm2), DR (557 cells/mm2) and NR (1111 cells/mm2) $p = 0.034$. Several PCAs were unable to identify a signature associated with recurrence pattern.

Conclusion:

The biomarkers used in this study were helpful in distinguishing tumor from normal tissue and high Ki-67 may be associated with LOR, however, a useful molecular signature for recurrence pattern could not be identified.

Introduction

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 from patient tumors and normal bladder tissue using quantitative immunohistochemistry (IHC) and immunofluorescence (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.

Materials 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 (Fig. 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) on the BOND RX autostainer (Leica Biosystems, Vista, CA). 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.

Quantitative image analysis

Multi-layer TIFF images were exported from InForm (PerkinElmer) and loaded into HALO (Indica Labs, New Mexico) 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. 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 per core x100), 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 analyses were performed to access 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 1. 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.

Table 1
Patient characteristics.

Age	Number (n = 42)	Percent (%)
Median	70	
Range	53–85	
Sex		
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%

Tumor vs normal tissue

There were notable differences between tumor and normal tissue with regard to biomarker expression (Table 2, Fig. 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.

Table 2
Tumor vs normal tissue values for Ki-67, MRE11, and PD-L1 biomarkers with comparative two-tailed t-tests. Significant p-values ($p < 0.05$) are in bold.

Molecular variable	Mean for normal	Mean for tumor	Tumor compared to normal	t-test p-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 20X field	912	2319	Increased	0.060
% MRE11 Positive Cells	66.2	52.8	Decreased	0.21
MRE11 Positive Cell density (cells/mm2)	1355	2749	Increased	0.17
Ki67 Positive Cells per 20X field	764	923	Increased	0.84
% Ki67 Positive Cells	39.0	22.2	Decreased	0.60
Ki67 Positive Cell density (cells/mm2)	1183	1115	Decreased	0.96
PD-L1 Positive Cells per 20X field	35	237	Increased	**0.019**
% PD-L1 Positive Cells	2.00	4.98	Increased	0.12
PD-L1 Positive Cell density (cells/mm2)	53.4	274	Increased	**0.034**

Associations with the pattern of recurrence and survival

On multivariate regression, positive surgical margin was the only variable associated with increased risk of recurrence ($t = 2.14$, $p = 0.041$). On student's t-test (univariate analysis), three Ki-67 IF measurements were associated with a higher likelihood of LOR versus DR: Ki-67 positive cells ($p = 0.036$), percent Ki-67 positive cells ($p = 0.026$), and Ki-67 positive cell density ($p = 0.034$) (Table 3). Ki-67 cell density varied by recurrence type: LR (1354 cells/mm2), DR (557 cells/mm2) and NR (1111 cells/mm2) ($p = 0.034$). On multivariate Cox PH modeling the following variables were associated with worse overall survival: 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). The following were associated with lower recurrence free survival: 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.

Table 3
Two-tailed t-tests for recurrence patterns based on Ki-67, MRE11, and PD-L1 expression.

test on	Mean for NR	Mean for LOR	mean for DR	t-tests p-values		
				none vs. LOR	none 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 20X field	2301	2394	2193	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/mm2)	2676	2926	2530	0.77	0.77	0.44
Ki67 Positive Cells per 20X field	946	1084	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/mm2)	1111	1354	577	0.59	0.082	**0.034**
PD-L1 Positive Cells per 20X 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/mm2)	180	397	231	0.34	0.83	0.58

NR = no recurrence, LOR = local only recurrence, DR = distant recurrence

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 (Fig. 2), the second included all 22 clinical and biomarker variables, and the third was limited to margin status and K-

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 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, in MIBC tumors compared to surrounding bladder tissue (Table 2, Fig. 1). These findings are consistent with known hallmarks of cancer including increased cellular proliferation and evasion of immune system detection. Second, our data show that high Ki-67 cell density may be associated with increased rates of local versus distant recurrence (Table 3). 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 [14–17]. Our data also suggest that tumors with high Ki-67, while associated with higher rates 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, 18–19], 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.

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.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board at the University of Southern California (IRB#: HS-01B014-CR015). There were no human or animal subjects. Ethics committee approval and consent from participants was not required.

Consent for publication

Not applicable

Availability of data and materials

Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

Competing interests

Dr. Magliocco reports personal fees from Protean BioDiagnostics Inc, outside the submitted work. Dr. Mouw reports grants from Pfizer, personal fees from UpToDate, personal fees from OncLive, outside the submitted work.

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Authors' contributions

Conception: LKB, AM, SD

Performance of work: YX, MA, ZM, SM, CP,

Interpretation or analysis of data: CCF, LKB, YX, AM

Writing the article: CCF, LKB, AM, SD, KWM, TD, SKB

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Figures

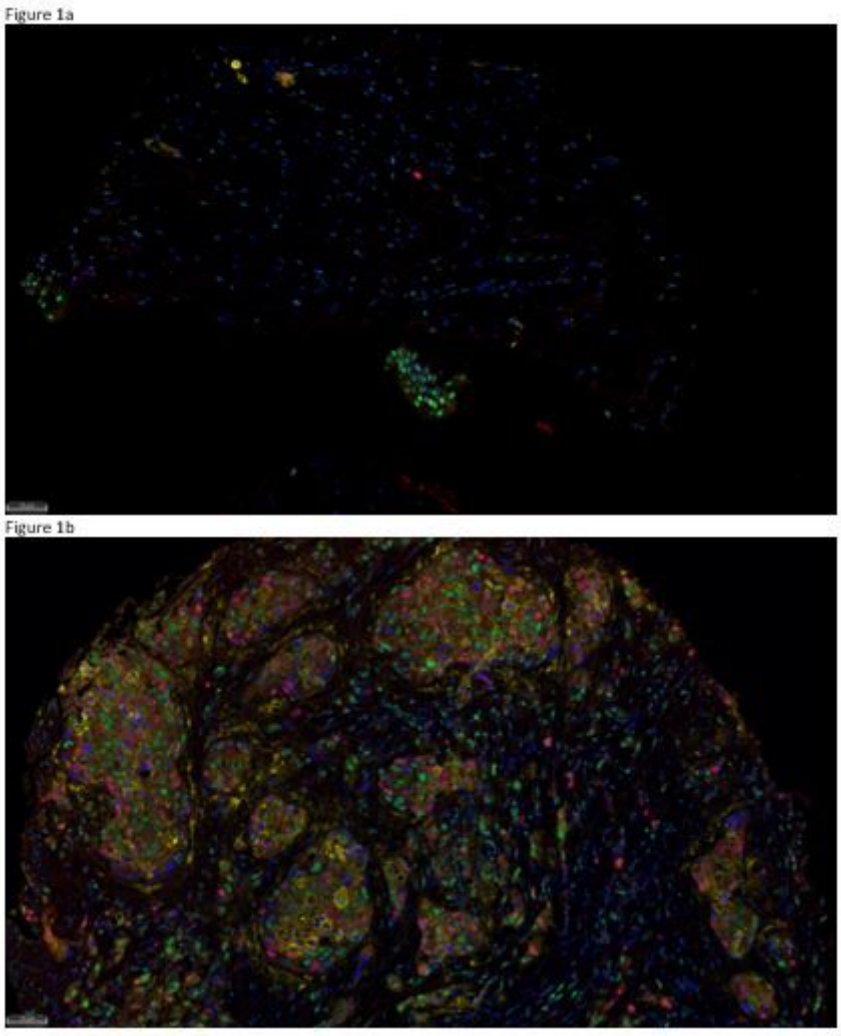

Figure 1

Immunofluorescence of normal tissue (1a) and tumor (1b) for MRE11 (green), PD-L1 (yellow), and Ki-67 (red) using Vectra 3 scans at 20X magnification. Blue represents DAPI nuclear stain.

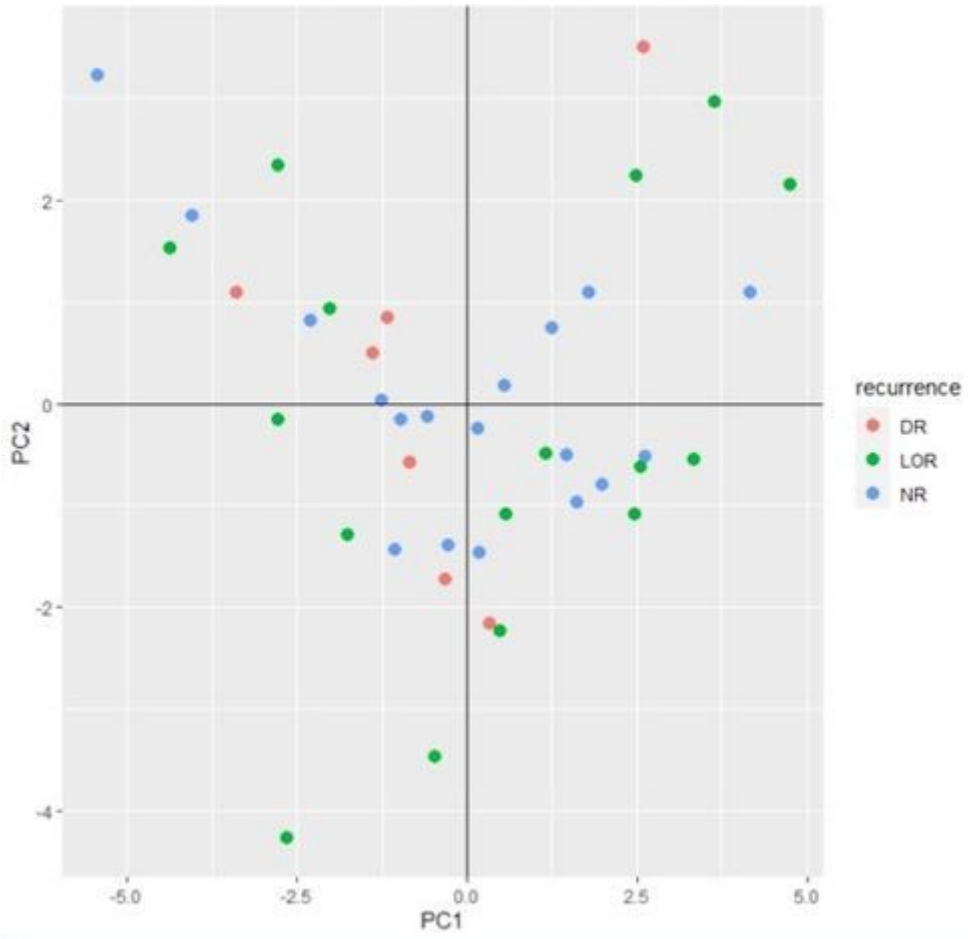

Figure 2

Multidimensional scaling map depicting a principal component analysis (PCA) of all 15 biomarker variables. None of the PCAs used in this study were effective in separating patient samples by recurrence patterns (LOR = local only recurrence, DR = distant recurrence, NR = no recurrence).