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ORIGINAL ARTICLE

Melanoma-associated antigen-A and programmed death-ligand 1 expression are associated with advanced urothelial carcinoma

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Abstract

Background Melanoma-associated antigen-A (MAGE-A) and programmed-death ligand 1 (PD-L1) are present in urothelial carcinoma (UC). We assessed survival outcomes in patients with MAGE-A and PD-L1 expression.

Methods MAGE-A and PD-L1 expression on neoplastic cells was analyzed using tissue microarrays from patients with UC. We compared differential expression between disease stage and grade. MAGE-A and PD-L1 co-expression was sub-categorized. Fisher's exact test was done for categorical variables followed by univariable and multivariable analysis of recurrence-free survival (RFS) and progression-free survival (PFS).

Results Co-expression of MAGE+/PD-L1+ was higher in advanced disease; however, only MAGE+/PD-L1− was associated with shorter RFS [hazard ratio (HR) 1.89; 95% confidence interval (CI) 1.19–2.99; $p = .006$]. MAGE+/PD-L1+ was associated with the worst PFS (HR 17.1; 95% CI 5.96–49.4; $p \leq .001$). MAGE-A expression was more prevalent with high-grade ($p = .015$), and higher-stage ≥ pT2 ($p = .001$) disease. The 5-year RFS was 44% for MAGE+ versus 58% for MAGE− patients. On multivariable analysis, MAGE+ was also associated with shorter RFS (HR 1.55; 95% CI 1.05–2.30; $p = .03$). Similarly, MAGE+ was associated with shorter PFS (HR 3.12; 95% CI 1.12–8.68; $p = .03$).

Conclusion MAGE-A and PD-L1 expression is increased in advanced disease and associated with shorter PFS. Furthermore, MAGE-A expression was significantly associated with higher-grade and -stage disease and associated with shorter RFS and PFS. The worse prognosis associated with MAGE-A+/PD-L1+ provides evidence that a combinatorial treatment strategy co-targeting MAGE/PD-L1 might be feasible. Further studies are needed to validate these findings.

Keywords Urothelial carcinoma · Melanoma-associated antigen · Programmed death-ligand 1 · Tissue microarray · Survival

Abbreviations

CI	Confidence interval
HR	Hazard ratio
IQR	Inter-quartile range
MAGE-A	Melanoma-associated antigen-A
MP	MAGE-positive
MN	MAGE-negative
PD-L1	Programmed death-ligand 1
RC	Radical cystectomy
RFS	Recurrence-free survival
TCR	Adoptive T-cell receptor-engineered T-cell therapy
TMA	Tissue microarray
UC	Urothelial carcinoma

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Introduction

The melanoma-associated antigen-A (MAGE-A) gene family consists of 12 MAGE-A genes located on chromosome Xq28. The function and biological role of MAGE-A proteins

in cancer have not been completely elucidated; however, members of MAGE-A have been implicated in modulating the activity of E3 ubiquitin ligases on targets related to apoptosis and in the suppression of p53-dependent apoptosis [1]. MAGE has been a highly attractive target for cancer immunotherapy because of its broad representation in cancer tissues, but restricted expression in normal adult tissues, namely, immune-privileged germ cells. Recent studies have shown significant expression of MAGE antigen in urothelial carcinoma (UC) [2–6]. This observation has clinical implications as other studies have shown a poor prognosis in MAGE-positive patients [6, 7]. Currently, there are numerous approaches targeting this antigen in the clinical setting, including vaccines and adoptive T-cell receptor-engineered T-cell therapy (TCR). A recent phase I trial using a TCR targeting MAGE-A has shown a potential benefit [8]. The safety of this TCR across multiple tumor types is currently being evaluated in a phase I trial (NCT03139370).

The use of immune checkpoint inhibitors in UC has become an important salvage option with reasonable response rates for patients whose disease progresses on cytotoxic chemotherapy [9]. While MAGE-A expression has been described in UC, evidence is lacking regarding the correlation of MAGE and programmed death-ligand 1 (PD-L1) expression in UC. These data are of interest, as they inform a potential combinatorial therapeutic strategy using adoptive cell transfer together with a checkpoint inhibitor. It is widely recognized that the use of TCRs in solid tumors will likely require combination therapy to address an immunosuppressive tumor microenvironment, such as in combination with checkpoint inhibitors, for example [10]. Some early data suggest that a combination approach may potentiate an immune response and enhance the efficacy of using adoptive cell therapy [11–13]. In addition, there are studies that suggest that receiving checkpoint inhibitors prior to tumor-infiltrating lymphocyte (TIL) harvest may lead to more effective TIL harvest, with a shorter ex vivo expansion time, and increased efficacy [14, 15]. In this study, we aimed to assess survival outcomes in patients with UC and their correlation with MAGE and PD-L1 expression.

Materials and methods

Study population and outcomes

The study cohort consisted of 422 UC samples in 275 patients from transurethral resection of bladder tumor or radical cystectomy (RC) done at a tertiary medical center between 1985 and 1998. Available clinical, pathological, and follow-up data on each patient were obtained. The main covariates were age, sex, race, smoking history, cancer history, procedure, pathologic stage, and grade. The study outcomes were recurrence-free survival (RFS) and progression-free survival (PFS) in patients who underwent both RC and transurethral resection of bladder tumor, and overall survival (OS) in patients who underwent RC only. The overall aim was to examine the association of MAGE expression with survival outcomes as well as MAGE and PD-L1 expression with survival outcomes.

Tissue microarray construction

Tissue from formalin-fixed, paraffin-embedded specimens was obtained. Three 0.6-mm core biopsies were taken from representative tumor regions and precisely arrayed using a custom-built instrument as previously described [16]. 4-µm sections of the tissue microarray block were transferred to glass slides using the paraffin sectioning aid system comprising adhesive-coated PSA-CS4x slides, adhesive tape, and an ultraviolet lamp (InstruMedics, LLC, Hackensack, New Jersey) to support the cohesion of 0.6-mm array elements.

Immunohistochemical staining and evaluation

Immunohistochemical (IHC) analysis of MAGE-1 (mouse clone 6C1, Thermo Fisher Scientific, Waltham, MA), and PD-L1 (rabbit clone SP142, Spring Biosciences) was performed at room temperature on the Dako Link Autostainer 48 (Agilent, Santa Clara, CA) [17] (Supplementary figure 1). Tissue sections were pretreated using Rip Tide, a proprietary antigen retrieval buffer (Mosaic Laboratories, Lake Forest, CA) for 40 min at 95 °C. Once the Autostainer procedure was initiated, the slides were rinsed with buffer immediately and after each of the following steps: (1) incubation with Envision Peroxidase (Dako) for 5 min to quench endogenous peroxidase; (2) incubation with MAGE-1 antibody, PD-L1 antibody, or isotype-negative control for 30 min; (3) detection with Envision FLEX Linker for 15 min; (4) detection with Envision FLEX horseradish peroxidase for 20 min; and (5) staining with diaminobenzidine (Dako) for 10 min each. Upon completion of the staining procedure, slides were counterstained offline with hematoxylin (Dako) for 2 min, rinsed, and coverslipped.

Evaluation of IHC stains was performed by a pathologist who recorded the staining intensity, subcellular localization, and percentage of positively stained tumor cells. Staining intensity was evaluated on a semi-quantitative scale with the percentage of cells stained at each of the following four levels recorded: 0 (unstained), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong staining). An H-Score was calculated based on the summation of the product of percentage of cells stained at each intensity using the following equation: (3 × percentage of cells stained at 3+) + (2 × percentage of cells stained at 2+) + (1 × percentage of cells stained at 1+). The maximum staining intensity of

normal adjacent tissue, endothelia, smooth muscle, fibroblasts, stroma, inflammatory cells, and nerve were recorded if observed. If positive PD-L1 staining was observed in endothelial cells, the percentage of staining was estimated. MAGE-positive (MP) status was defined as ≥ 50% positive staining with 2 + or 3 + intensity, while MAGE-negative (MN) was defined as below this level, a threshold that has been used previously in evaluating MAGE staining [8]. A commonly used PD-L1 cutoff criterion of ≥ 1% positive staining of tumor cells was applied [18].

Statistical analysis

Descriptive statistics for study variables were computed for the overall cohort as well as for the MP and MN subgroups. Study variables were compared between these subgroups using the Wilcoxon rank-sum tests for continuous variables and Chi-square or Fisher's exact tests for categorical variables. We further subdivided patients according to MAGE and PD-L1 staining status to assess the effect of co-expression on survival. Patients' specimen-level data were used for the analysis for recurrence, as biopsies were done at each recurrence, thus reflecting this particular outcome, with follow-up time calculated from procedure until event or censoring. However, patient-level data were used to assess PFS and OS. RFS curves were created with the Kaplan–Meier method and survival was compared between discrete MAGE and PD-L1 expression groups with the log-rank test. For recurrence, univariable and multivariable mixed-effects Cox proportional hazards model were used to evaluate predictors of outcome, adjusting for repeated measurements per patient. Variables selected in the model were either significant on the univariable analysis and/or were felt to be clinically relevant. However, due to small number of events for progression, only a univariable analysis was conducted. Tests for proportionately were not violated in all models. OS was analyzed only in patients who underwent RC using Cox models. Performance of the Cox models was assessed using Harrell's C statistic [19]. A sensitivity analysis was also performed to assess effect of year of treatment on OS. Two-tailed p values < 0.05 were considered statistically significant. Statistical analyses were performed with Stata statistical software version 15 (StataCorp, LLC, College Station, TX, USA).

Results

Patient characteristics

The patient cohort consisted of 275 patients and 422 samples (Tables 1, 2). Median age was 70 [inter-quartile range (IQR) 62–76] with the majority of the cohort consisting of male, Caucasian patients. A large proportion of patients were smokers or former smokers (54%). MAGE staining was associated with more advanced disease stage (≥ pT2 52% versus 42%) and high-grade disease (73% versus 60%; $p = .015$). A similar trend was noted for PD-L1-positive samples, which were more likely to be higher disease stage and grade.

Table 1 Baseline characteristics

	Total ($N = 275$)
Age	70 (62–76)
Sex	
Male	223 (81)
Female	52 (19)
Ethnicity	
Caucasian	235 (85)
AA	6 (2)
Other	34 (12)
Tobacco	
No	29 (11)
Yes	149 (54)
Unknown	97 (35)
Personal history of other cancers	
No	148 (54)
Yes	52 (19)
Missing	75 (27)
Procedure	
Transurethral resection of bladder tumor	120 (44)
Radical cystectomy	136 (49)
Other	19 (7)

MAGE outcomes

The median follow-up time for the entire cohort was 77 months (IQR 22–118 months). The median RFS for patients with MP samples was 32 months, while those with MN samples had a median RFS that was not reached. The 5-year RFS in the MP group was 44% compared with 58% in the MN group (Fig. 1). In a univariable Cox model (Table 3), MP was significantly associated with recurrence [hazard ratio (HR) of 1.84; 95% confidence interval (CI) 1.09–3.09; $p = .02$]. Similarly, on multivariable analysis, adjusting for baseline and clinical variables, MP was also associated with shorter recurrence (HR of 1.55; 95% CI 1.05–2.30; $p = .03$). Model performance using Harrel's C statistic was 0.64. Median follow-up for patients who underwent RC was 38 months (IQR 13–101). The 5-year PFS in the MP group was 44% compared with 82% in the MN group (Fig. 1). On univariable analysis, MP was also significantly associated with shorter PFS (HR of 3.12; 95% CI 1.12–8.68; $p = .03$). The median OS in patients who underwent RC and

Table 2 Tumor and clinical characteristics—MAGE

				MAGE and PD-L1 subgroups				
	MAGE Neg	MAGE Pos	*p* value	MN/PN	MP/PN	MN/PP	MP/PP	*p* value
Overall, *n* (%)	321 (76)	101 (23)		232 (55)	72 (17)	89 (21)	29 (7)	
Target stage			< 0.001					< 0.001
pTa	113 (36)	21 (21)		96 (42)	17 (24)	17 (19)	4 (14)	
pTis	34 (11)	4 (4)		27 (12)	3 (4)	7 (8)	1 (3)	
pT1	38 (12)	23 (23)		25 (11)	17 (24)	13 (15)	6 (21)	
pT2	61 (19)	33 (33)		35 (15)	20 (28)	26 (30)	13 (45)	
pT3–4	50 (16)	13 (13)		30 (13)	10 (14)	20 (23)	3 (10)	
Metastasis	21 (7)	6 (6)		16 (7)	4 (6)	5 (6)	2 (7)	
Grade			0.015					< 0.001
Low	129 (40)	27 (27)		109 (47)	24 (33)	20 (22)	3 (10)	
High	192 (60)	74 (73)		123 (53)	48 (67)	69 (78)	26 (90)	
Surgical margins			0.92					0.72
Negative	130 (40)	42 (42)		90 (39)	29 (40)	40 (45)	13 (45)	
Positive	13 (4)	3 (3)		10 (4)	1 (1)	3 (3)	2 (7)	
Missing	178 (55)	56 (55)		132 (57)	42 (58)	46 (52)	14 (48)	

had samples with MP staining was 46 months compared with 77 months in patients with MN samples. In addition, 5-year OS was 44% versus 51% in the MP versus MN group, respectively (Fig. 2). However, there was no association with OS in either the univariable (HR of 1.15; 96% CI 0.71–1.87; $p = .56$) or multivariable Cox model (HR of 1.01; 95% CI 0.58–1.75; $p = .97$). Furthermore, in a sensitivity analysis, year of treatment did not influence OS.

MAGE/PD-L1 outcomes

There were 27% PD-L1–positive samples. Co-expression of MAGE and PD-L1 was assessed by categorizing samples in subgroups by staining result (Table 2). A high proportion of non-muscle-invasive bladder cancer samples (65%) were negative for both MAGE and PD-L1; whereas, there was a significant association of higher-stage disease ($p \leq .001$) with samples that were positive for either marker. In the MP/PD-L1-negative group, 48% were ≥ pT2; whereas, in the MN/PD-L1-positive and double MP/PD-L1-positive groups, the percentages were 59% and 62%, respectively. A similar trend was observed for disease grade; 90% of samples that were positive for both MAGE and PD-L1 were high-grade ($p \leq .001$). On univariable analysis, overall PD-L1 positivity was not associated with shorter RFS (HR of 0.79; 95% CI 0.55–1.14; $p = .21$). However, PD-L1 positivity was associated with shorter PFS (HR of 5.31; 95% CI 1.99–14.2; $p = .001$) (Table 4). MP/PD-L1–negative samples comprised a subset associated with a shorter median RFS of 19 months (HR of 1.96; 95% CI 1.30–2.95; $p \leq .001$) (Fig. 2). Furthermore, when adjusting for clinical variables, this subset was still associated with shorter RFS (HR of 1.89; 95% CI 1.19–2.99; $p = .006$) (Table 5). Model performance using Harrell's C statistic resulted in an area under the curve of 0.65. Of the associations observed, the most significant was that of the double-positive subset with shorter PFS (HR of 17.1; 95% CI 5.96–49.4; $p \leq .001$). There was no difference in RFS among the other groups (Table 4). OS was also not significantly different between the groups (Fig. 2).

Discussion

The treatment of UC has undergone major changes in recent years, namely the addition of immuno-oncology agents that have been shown to provide significant benefit in advanced disease [20]. While excitement is warranted, curative therapies for advanced disease are lacking. Cancer/testis antigens, specifically MAGE-A, have emerged as potential targets for immune-oncologic strategies in the setting of advanced cancers. The prognostic value of MAGE-A expression has been established in numerous malignancies without evidence of expression in normal tissue [21]. In UC, a number of studies using different expression analyses have shown that a significant proportion of patients with UC have expression of MAGE-A, and increased expression of MAGE-A is associated with shorter clinical outcomes. Dyrskjøt et al. have shown, using quantitative reverse-transcriptase polymerase chain reaction, that 43% of tumors showed MAGE-A expression and were associated with shorter PFS (HR of 2.96; 95% CI 1.14–7.68; $p = .026$) [4]. Similarly, the expression profile of MAGE-A using tissue microarray in a large cohort demonstrated an association of MAGE-A expression with shorter cancer-specific survival in UC (HR of 1.44, 95% CI 1.05–1.99; $p = .02$) [6]. In our study, using a more restrictive definition of MP using IHC, we found increased expression

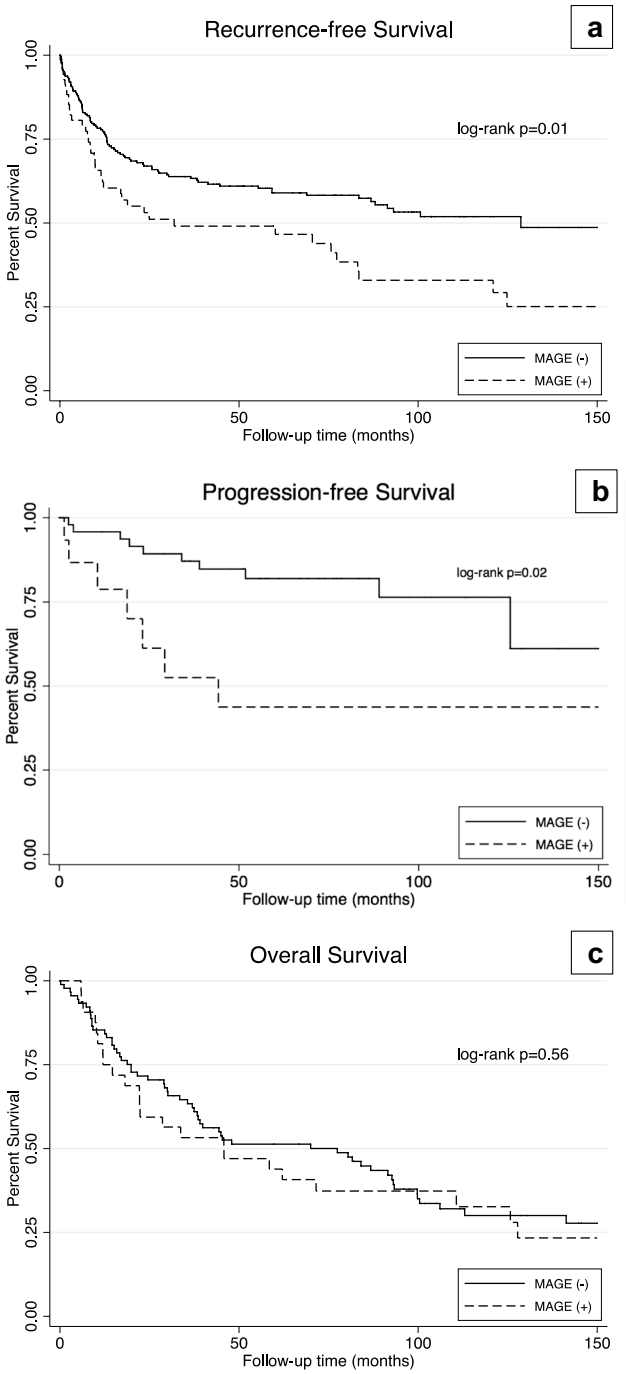

Fig. 1 Recurrence-free (**a**), progression-free (**b**), and overall survival (**c**) outcomes for MAGE-A staining (cutoff ≥ 50% positive staining with 2+/3+ intensity)

of MAGE-A in 23% of samples, which was significantly associated with shorter RFS with both the univariable and multivariable survival model. In addition, PFS was significantly shorter in the MP group versus the MN group. In an exploratory analysis, we also found 43% of the samples with UC were positive for MAGE-A expression using a less restrictive definition, consistent with previous studies. Furthermore, OS was not significantly different between the MP and MN groups. This may be partly due to comorbidities or various treatments received that affect OS.

The possibility of using a combination strategy to target tumors, unleashing the immune system by targeting MAGE-positive tissue while simultaneously allowing the immune system to function unimpeded via PD-L1 blockade, is an attractive one [22]. Such strategies are being evaluated in other malignancies and potentiated responses have been shown using combination approaches in mouse models [11, 12]. Clinical trials using a combination of adoptive cell and checkpoint inhibitor therapy are underway (NCT03296137, NCT03287674, NCT03296137). Thus, further exploration of MAGE and PD-L1 expression patterns in UC is warranted as a foundation for such combination approaches. In our secondary analysis, we explored the prognostic implications of both MAGE-A and PD-L1 expression, and whether it informs such a strategy in UC. To assess co-expression, we subcategorized expression groups. The MP/PD-L1-negative group was associated with shorter RFS compared with the other expression groups. The MP/PD-L1 double-positive group was significantly associated with shorter PFS, but was not associated with shorter RFS, despite the fact that 62% of samples in this group were from patients with ≥ pT2 stage disease and 90% were from patients with high-grade disease (Table 2), features that would be expected to be associated with recurrence. This may be partially explained by the fact that many recurrences may have occurred in patients with lower-stage disease, and that MAGE-A and PD-L1 expression seems to be more prevalent in more advanced disease.

These results must be interpreted within the strengths and limitation of the study. The strengths of this study are first, the ability to study a relatively large set of samples from UC, while looking at the recurrence and progression outcomes in association with MAGE-A and PD-L1 expression. Second, to our knowledge, there are no studies examining prognostic implications of the co-expression of MAGE-A and PD-L1 in UC. While it is unclear whether expression of MAGE-A will influence PD-L1 expression or vice versa, this question is clinically relevant because there is a reasonable proportion of patients whose tumors simultaneously express MAGE and PD-L1, perhaps supporting a possible combination strategy. In addition, this study used uniform tissue microarray construction, staining, and interpretation to reduce potential variability. The limitations of this study include its retrospective nature as well as many potential confounders and variables across patients, such as performance status and number of treatments received, which were not controlled for and may affect the results. In addition, despite our attempts to address the heterogeneity of the cohort by statistical methods, this may still represent an important limitation. Conversely, this was seen as an opportunity to interrogate the sub-population

Table 3 Recurrence-free survival model—MAGE

	Unadjusted		Adjusted[a]	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
MAGE				
Negative	Ref.		Ref.	
Positive	1.67 (1.18–2.38)	0.004	1.62 (1.09–2.40)	0.02
PD-L1				
Negative	Ref.		Ref.	
Positive	0.79 (0.55–1.14)	0.21	0.80 (0.53–1.21)	0.29
Age	1.02 (0.99–1.03)	0.06	1.01 (0.99–1.03)	0.09
Sex				
Male	Ref.		Ref.	
Female	0.85 (0.54–1.32)	0.47	0.82 (0.52–1.30)	0.40
Ethnicity				
Caucasian	Ref.		Ref.	
AA	1.23 (0.64–2.34)	0.53	1.38 (0.72–2.67)	0.33
Other	1.85 (1.29–2.64)	0.001	1.68 (1.05–2.71)	0.03
Tobacco				
No	Ref.		Ref.	
Yes	0.54 (0.29–0.99)	0.047	0.60 (0.31–1.16)	0.12
Unknown	0.85 (0.46–1.57)	0.61	0.56 (0.26–1.14)	0.12
History of other cancers				
No	Ref.			
Yes	1.11 (0.67–1.84)	0.68		
Missing	1.88 (1.31–2.71)	0.001		
Target stage				
pTa	Ref.		Ref.	
pTis	1.12 (0.66–1.87)	0.67	1.11 (0.52–2.35)	0.79
pT1	1.47 (0.90–2.39)	0.12	1.28 (0.69–2.39)	0.43
pT2	0.81 (0.51–1.29)	0.38	0.71 (0.34–1.49)	0.36
pT3–4	0.74 (0.38–1.42)	0.37	0.81 (0.32–2.08)	0.67
Metastasis	1.05 (0.54–2.04)	0.89	0.79 (0.33–1.90)	0.60
Grade				
Low	Ref.		Ref.	
High	1.01 (0.73–1.39)	0.97	1.34 (0.70–2.61)	0.37
Surgical margins				
Negative	Ref.		Ref.	
Positive	0.82 (0.36–1.86)	0.63	0.91 (0.37–2.24)	0.84
Missing	0.85 (0.39–1.87)	0.70	1.47 (0.90–2.40)	0.12

[a]Adjusted for age, sex, race, smoking history, pathologic stage, grade, and margins

of patients using different endpoints and analyses, especially the prognostic value of MAGEA staining in TURBT samples. Finally, given the exploratory, hypothesis-generating nature of this study, the results remain valid in providing preliminary data to build on in future studies.

Conclusion

In this study, we demonstrate the association of MAGE-A IHC expression in UC with shorter RFS and PFS.

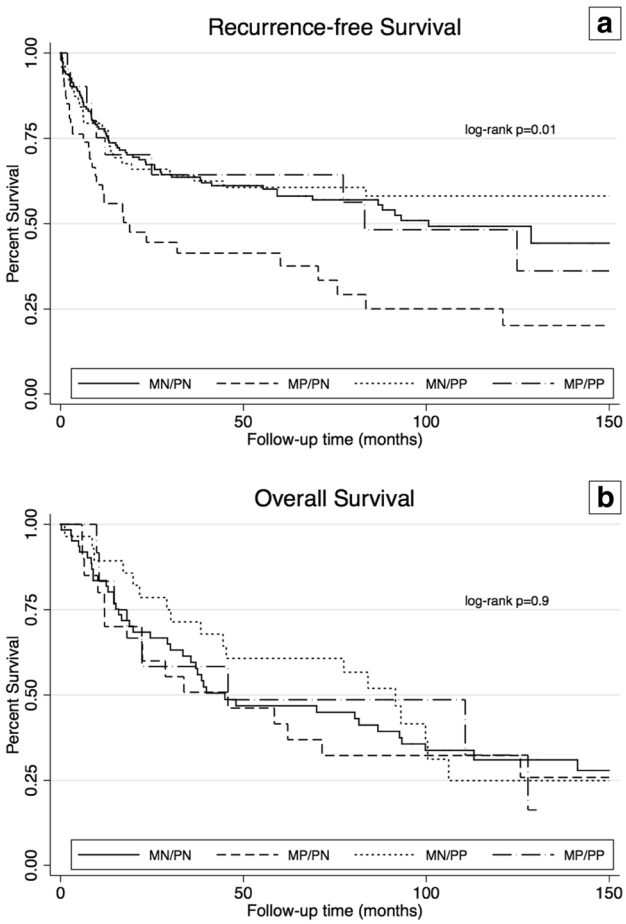

Fig. 2 Recurrence-free (**a**) and overall survival (**b**) outcomes for MAGE-A (cutoff ≥ 50% positive staining with 2+/3+ intensity) and PD-L1 co-expression (cutoff ≥ 1% staining)

Table 4 Progression-free survival model

	Unadjusted	
	HR (95% CI)	*p* value
MAGE		
Negative	Ref.	
Positive	3.12 (1.12–8.68)	0.03
PD-L1		
Negative	Ref.	
Positive	5.31 (1.99–14.2)	0.001
Groups		
MN/PN	Ref.	
MP/PN	4.16 (0.86–20.1)	0.08
MN/PP	6.62 (1.99–22.0)	0.002
MP/PP	17.1 (5.96–49.4)	< 0.001

Furthermore, co-expression of MAGE-A and PD-L1 are present in more advanced disease states, which translated into shorter PFS. This supports the possibility of co-targeting of MAGE and PD-L1 in advanced UC. Further study and validation of these findings are warranted.

Table 5 Recurrence-free survival model—MAGE/PD-L1

	Unadjusted		Adjusted[a]	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Groups				
MN/PN	Ref.		Ref.	
MP/PN	1.96 (1.30–2.95)	0.001	1.89 (1.19–2.99)	0.006
MN/PP	0.88 (0.56–1.38)	0.57	0.96 (0.59–1.55)	0.86
MP/PP	1.07 (0.58–1.99)	0.82	1.04 (0.52–2.07)	0.92

[a]Adjusted for age, sex, race, smoking history, pathologic stage, grade, and margins

Author contributions IF: Conception, data collection, data analysis, drafting manuscript, critical revisions. SHA: Conception, drafting manuscript, critical revisions. DAE: Data analysis, drafting manuscript, critical revisions. RJ: Drafting manuscript, critical revisions. AB: Conception, drafting manuscript, critical revisions. KC: Conception, drafting manuscript, critical revisions. ASB MD: Conception, critical revisions. AJP: Conception, drafting manuscript, critical revisions. AD: Conception, drafting manuscript, critical revisions.

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Compliance with ethical standards

Conflict of interest Izak Faiena and Alexandra Drakaki received research funding from Kite, a Gilead Company. Stephanie H. Astrow, Rajul Jain, and Adrian Bot are employees of Kite, a Gilead Company, and have equity ownership in Gilead Sciences, Inc. Arie S. Belldegrun is the founder and was formerly Chief Executive Officer of Kite, a Gilead Company, and has equity ownership in Gilead Sciences, Inc. Allan J. Pantuck has equity ownership in Gilead Sciences, Inc. The authors declare they have no other conflicts of interest.

Ethical approval and ethical standards This study included de-identified data in a tissue microarray and was deemed to be an exempt study by the institutional review board (IRB #99–233) of the University of California, Los Angeles for TMA construction and data analysis; therefore, special ethical permission was not required. Requirement for consent was waived given the retrospective, de-identified nature of the samples, and the impracticality of consenting for samples stored prior to 1998.

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