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Tumor LAG-3 and NY-ESO-1 expression predict durable clinical benefits of immune checkpoint inhibitors in advanced non-small cell lung cancer

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Abstract

Background: Immune checkpoint inhibitors (ICIs) are an established treatment for non-small cell lung cancer (NSCLC) that have demonstrated durable clinical benefits (DCBs). Previous studies have suggested NY-ESO-1 and LAG-3 to be surrogate markers of ICI responses in NSCLC; therefore, we explored the predictive value of their expression in NSCLC.

Methods: We retrospectively reviewed the records of 38 patients with advanced NSCLC treated with anti-PD-1 monoclonal antibodies from 2013 to 2016 at Seoul National University Hospital and Seoul National University Bundang Hospital after failed platinum-based chemotherapy. Tumor tissues from each patient were subjected to immunohistochemical analysis to determine NY-ESO-1, LAG-3, and PD-L1 expression, whose ability to predict progression-free survival (PFS) and overall survival (OS) was then analyzed alongside their positive (PPV) and negative (NPV) predictive values.

Results: NY-ESO-1 or LAG-3 expression was detected in all tumor samples from patients with high PD-L1 expression and was significantly associated with favorable outcomes, unlike PD-L1 expression. Patients with both NY-ESO-1- and LAG-3-expressing tumors had a high DCB rate and those with triple-positive PD-L1, LAG-3, and NY-ESO expression had a superior median OS and PFS than those with triple-negative expression. Furthermore, LAG-3 and NY-ESO-1 co-expression was an independent predictor of both PFS and OS, while LAG-3 displayed a good NPV.

Conclusions: Patients with NSCLC who co-express NY-ESO-1 or LAG-3 with PD-L1 exhibit greater DCBs and improved long-term survival following anti-PD-1 therapy. Moreover, NY-ESO-1 and LAG-3 could be novel predictive biomarkers of survival and should be considered in the future use of ICIs.

KEYWORDS
immune checkpoint inhibitor, LAG-3, non-small cell lung cancer, NY-ESO-1, PD-L1

INTRODUCTION

Immune checkpoint inhibitors (ICIs) are an established standard of care for non-small cell lung cancer (NSCLC) as

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they have demonstrated durable clinical benefits (DCBs) in many clinical trials. Recent American Society of Clinical Oncology (ASCO) guidelines have recommended treatment with ICIs either alone or in combination with chemotherapy for nonsquamous NSCLC without epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) alteration and with high programmed cell death ligand 1 (PD-L1) expression (tumor proportion score [TPS] >50 %).[1] The Checkmate-227 clinical trial revealed that patients with chemo-naïve NSCLC with PD-L1 expression (TPS >1%) who received dual checkpoint blockade with nivolumab and ipilimumab displayed greater survival benefits than those who received platinum-based chemotherapy.[2] Based on the mode of action of ICIs, PD-L1 expression was proposed as a logical predictor of clinical outcomes and has been recommended as a biomarker to guide treatment decisions. In some cases, however, expression of PD-L1 was not directly related to the good ICI response. Patients without PD-L1 expression also responded to ICI treatment. PD-L1 was found to be imprecise as a marker for identifying responsive patients and further research is underway to define subgroups who do not benefit from, or are refractory to, ICIs.[3]

Cancer/testis antigens (CTAs) are characterized by spontaneous immunogenicity and distinct expression patterns that are normally restricted to germ cells of the testis and placenta but are frequently activated in tumor cells. New-York esophageal squamous cell carcinoma 1 (NY-ESO-1) is considered to be the most immunogenic CTA and studies have reported that its expression in NSCLC is related to poor prognosis following chemotherapy.[4,5] In addition, Matsuzaki et al. reported that tumor-derived NY-ESO-1-specific CD8+ T cells display impaired effector function and enriched PD-L1 expression in patients with ovarian cancer[6] and suggested that programmed cell death protein 1 (PD-1) blockade may augment their proliferation and cytokine production. Moreover, ipilimumab treatment has demonstrated clinical benefits in patients with melanoma who developed CD4+ and CD8+ peripheral blood lymphocytes with specificity against the NY-ESO-1 antigen,[7] while a phase II clinical trial of ipilimumab in patients with advanced melanoma and a spontaneous preexisting immune response to NY-ESO-1 revealed encouraging activity (immune-related partial response: 22.7%, immune-related stable disease: 27.3%).[8] Together, these studies suggest that NY-ESO-1 could be a good surrogate marker of ICI responses in NSCLC.

Lymphocyte activation gene-3 (LAG-3) is a checkpoint molecule that has been suggested as a targetable immunoregulatory molecule due to its negative regulatory roles in T cells. LAG-3 has also been found on a subset of activated T cells and studies have reported that tumor cell LAG-3 positivity is associated with poor prognosis.[9,10] In NSCLC, LAG-3 is mainly expressed on tumor-infiltrating T cells and correlates with PD-1/PD-L1 expression in tumor tissue.[10] Patients with NSCLC and high LAG-3 expression were also found to display poor survival after surgery, while combination anti-LAG-3 (BMS-986016) and anti-PD-1 (nivolumab) therapy has shown impressive clinical efficacy in patients with melanoma who are resistant to anti-PD-1/PD-L1 therapy.[11] Thus, LAG-3 may have predictive value in NSCLC.

Since both tumor-infiltrated T cells and the cancer-specific antigens that they recognize are crucial for generating effective anticancer immune reactions after ICI administration, we aimed to assess the predictive value of NY-ESO-1 and LAG-3 for survival after ICI treatment and to identify patients with advanced NSCLC who may receive clinical benefits from PD-1 antibody treatment.

METHODS

Patient selection

We retrospectively reviewed the medical and pathology records of 38 patients with advanced NSCLC who were treated with anti-PD-1 monoclonal antibodies from October 2013 to April 2016 at the Departments of Hemato-Oncology of Seoul National University Hospital (SNUH) and Seoul National University Bundang Hospital (SNUBH) after failed platinum-based chemotherapy. Lung cancer stage was categorized according to the TNM staging system (eighth edition). Patient inclusion criteria were as follows: (i) pathologically confirmed NSCLC; (ii) initial stage IIIB or IV, or recurrence after curative surgery; and (iii) received nivolumab or pembrolizumab as a palliative therapy. All patients received anti-PD-1 antibodies as participants in clinical trials (NCT01295827, NCT01905657, and NCT02175017). This study was approved by the Institutional Review Boards of SNUH and SNUBH (No. J-1607-085-776 and B-1606/349-110) and written informed consent was obtained from all participants according to the Declaration of Helsinki.

Immunohistochemical (IHC) analysis and cutoff determination

Tumor tissue was obtained from each patient at the time of diagnosis, acquired from surgical specimens and small biopsy specimens through percutaneous needle biopsy, bronchoscopic biopsy, and endoscopic bronchial ultrasonography biopsy. Specimens were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Additional IHC staining was carried out to determine tumor NY-ESO-1, LAG-3, and PD-L1 expression. The slides were treated according to standard protocol, fixed in neutral buffered formaldehyde, and processed into paraffin wax, and then incubated overnight with primary antibodies against NY-ESO-1 (E978, Invitrogen), LAG-3 (EPR4392, Abcam), and PD-L1 (22C3, Dako). Absence or presence of NY-ESO-1 expression on tumor cells and LAG-3 on immune cells were evaluated by an experienced lung cancer pathologist (Professor JH Chung). Semiquantitative assessment was performed by estimating

the percentage of positive cytoplasmic staining (Fig S1). To predict progression-free survival (PFS) and overall survival (OS), a >5% cutoff was used for LAG-3 and NY-ESO-1, while 5% and 50% cutoffs were used to analyze PD-L1 expression.

Treatment and assessment

Patients were treated with ICI monotherapy (nivolumab at 2 mg/kg every two weeks or pembrolizumab at 2 mg/kg or 10 mg/kg every three weeks) as a palliative therapy. Adverse event (AE) severity and laboratory findings were graded according to National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.03). Chest and abdominopelvic computed tomography scans were performed every 8–12 weeks according to previously described protocols.[12–14] Disease progression was assessed based on clinicians' decisions. Treatment response was evaluated according to revised response evaluation criteria in solid tumors (RECIST) guidelines (version 1.1). Pseudo-progression was also assessed during the study period and was defined as an increase in the size of target lesions or the appearance of new lesions followed by a response.

Statistical analysis

All data were analyzed using SPSS version 22.0 and collected from databases from July 1, 2017. To compare baseline patient characteristics and clinicopathological findings with patient responses to anti-PD-1 antibody treatment, categorical variables were analyzed using Pearson chi-square tests and Fisher's exact test, whereas continuous variables were analyzed using the Mann–Whitney U test. PFS and OS were analyzed for all assigned patients using the Kaplan–Meier method and two-sided log-rank tests. PFS was defined as the time from the date of the first nivolumab or pembrolizumab treatment to the date of either disease progression or death. OS was defined as the time between the dates of the first anti-PD-1 antibody treatment to death from any cause. The prognostic value of each variable was evaluated using univariate Cox proportional-hazard regression analyses. Multivariate PFS and OS analyses were performed using variables that were significant in the univariate analysis. All statistics were two-sided and statistical significance was defined as a p-value of <0.05.

RESULTS

Patient characteristics

From October 2013 to April 2016, 38 patients were enrolled in this study and their characteristics are summarized in Table 1. The median age of the patients when receiving ICI treatment was 67.5 (range 43–80) and 29 (76.3%) of the patients were male. All patients were treated with single agent immunotherapy after platinum failure and patients had received a median of two lines of treatment before immunotherapy. A total of 20 (52.6%) patients received nivolumab and 18 (47.4%) received pembrolizumab. The median time between diagnosis and immunotherapy was 6.75 months (range 0.7–-44.9 months) and the majority of patients (n = 27, 71.0%) were current or ex-smokers. Tumor tissue samples were obtained from surgical specimens of 29 patients (76.3%) and small biopsy specimens of nine patients (23.7%). More than half of patients (n = 22, 57.9%) had adenocarcinoma, 12 (36.6%) had squamous cell carcinoma, one had adenosquamous cell carcinoma, one had large cell neuroendocrine carcinoma, and two had NSCLC not otherwise specified. Two patients had *EGFR*-activating mutations, one had an ALK rearrangement, and one had a *KRAS* mutation. The sites of distant metastasis were as follows: lungs for 22 patients (57.9%), bones for 13 patients (34.2%), central nervous system for six patients (15.8%), pleural cavity for 16 patients (42.1%), liver for seven patients (18.4%), and adrenal glands for four patients (10.5%).

Clinical outcomes according to NY-ESO-1, LAG-3, and PD-L1 expression

Tumor tissues from all 38 patients were evaluated for NY-ESO-1 and LAG-3 expression; however, PD-L1 expression could not be determined for two patients due to lack of tissue samples. There were 19 (50%) and 29 (76.3%) patients who tested positive for NY-ESO-1 and LAG-3, while 11 (28.9%) and seven (18.4%) patients displayed PD-L1 expression with cutoffs of 5% and 50%, respectively (Table S1). Of the seven patients with high PD-L1 expression (> 50%), four also expressed NY-ESO-1 and six also expressed LAG-3, while three also displayed high PD-1 expression and were confirmed as triple-positive for all markers. Conversely, five patients were found to display triple-negative expression (Figure 1).

Next, we investigated the treatment response of the 36 patients, observing partial responses and stable disease in 34.2 and 31.6% of the patients, respectively. Neither complete remission nor pseudo-progression were observed during anti-PD-1 therapy which had a median duration of 10.29 months (range 0–39.0 months); however, the median duration of anti-PD-1 therapy in patients with triple-positive expression was 23.3 months and that in patients with triple-negative expression was less than five months. A total of 16 patients (44.4%) showed a DCB which was defined as more than six months of anti-PD-1 blockade without any evidence of disease progression, of which only five patients had high PD-L1 expression (5/7 vs. 11/29, p = 0.109). The following factors were found to exert a positive effect on DCB: single-positive NY-ESO-1 expression (OR = 0.260, p = 0.049), single-positive LAG-3 expression (OR = 0.448, p = 0.003), double-positive NY-ESO-1 and LAG-3 expression (OR = 0.101, p = 0.002), and double-positive LAG-3 and PD-L1 expression (OR = 0.105, p = 0.026). PD-L1 expression correlated with DCB following anti-PD-1

TABLE 1 Baseline clinical characteristics

	N	PFS <6 months (*n* = 22)	PFS ≥6 months (*n* = 16)	OR (95% CI) χ^2 test *p*-value
Age, years (median, range)	67.5 (43–80)	65.0 (51–80)	67.5 (43–78)	0.984 (0.920–1.053) *0.648*
Sex				
Men	29 (76.3%)	18 (47.4%)	11 (28.9%)	
Women	9 (23.7%)	4 (10.5%)	5 (13.2%)	2.045 (0.450–9.294) *0.350*
Smoking				
Current or ex-smoker	27 (71.0%)	16 (42.1%)	11 (28.9%)	
Never-smoker	11 (28.9%)	6 (15.8%)	5 (13.2%)	0.825 (0.201–3.391) *0.790*
ECOG performance status				
1	33 (86.8%)	20 (52.6%)	13 (34.2%)	
0	5 (13.2%)	2 (5.2%)	3 (7.9%)	0.433 (0.063–2.958) *0.384*
Histology				
Squamous cell carcinoma	12 (31.6%)	7 (18.4%)	5 (31.2%)	
Nonsquamous cell carcinoma	26 (68.4%)	15 (39.5%)	11 (28.9%)	1.027 (0.257–4.108) *0.970*
PD-1 antibody				
Pembrolizumab	18 (47.4%)	8 (21.1%)	10 (26.3%)	
Nivolumab	20 (52.6%)	5 (13.1%)	15 (39.5%)	1.200 (0.330–4.360) *0.782*
IHC analysis				
NY-ESO-1				
Negative	19 (50.0%)	14 (36.8%)	5 (13.2%)	
Positive	19 (50.0%)	8 (21.1%)	11 (28.9%)	0.260 (0.066–1.020) *0.049*
LAG-3				
Negative	9 (23.7%)	9 (23.7%)	0 (0%)	
Positive	29 (76.3%)	13 (34.2%)	16 (42.1%)	0.448 (0.299–0.671) *0.003*
PD-L1 (5% cutoff)				
Negative	25 (65.8%)	16 (42.1%)	9 (23.7%)	
Positive	11 (28.9%)	4 (10.5%)	7 (18.4%)	0.321 (0.074–1.405) *0.124*
NA	2 (5.2%)	2 (5.2%)	0 (0%)	
PD-L1 (50% cutoff)				
Negative	29 (76.3%)	18 (47.4%)	11 (28.9%)	
Positive	7 (18.4%)	2 (5.2%)	5 (13.2%)	0.244 (0.040–1.484) *0.109*
NA	2 (5.2%)	2 (5.2%)	0 (0%)	
NY-ESO-1 and LAG-3				
Negative	23 (60.5%)	18 (47.3%)	5 (13.2%)	
Positive	15 (39.5%)	4 (10.5%)	11 (28.9%)	0.101 (0.022–0.459) *0.002*
NY-ESO-1 and PD-L1				
Negative	34 (89.5%)	21 (55.3%)	13 (34.2%)	
Positive	4 (10.5%)	1 (2.6%)	3 (7.9%)	0.206 (0.019–2.200) *0.159*
LAG-3 and PD-L1				
Negative	32 (84.2%)	21 (55.3%)	11 (28.9%)	
Positive	6 (15.8%)	1 (2.6%)	5 (13.2%)	0.105 (0.011–1.012) *0.026*
Previous lines of systemic treatment				
>2	9 (23.7%)	6 (15.8%)	3 (7.9%)	
≤2	29 (76.3%)	16 (42.1%)	13 (34.2%)	0.615 (0.128–2.950) *0.542*
Distant metastases at diagnosis				
Liver metastases				
Yes	7 (18.4%)	7 (18.4%)	0 (0%)	
No	31 (81.6%)	15 (39.5%)	16 (42.1%)	0.484 (0.336–0.696) *0.012*

(Continues)

TABLE 1 (Continued)

	N	PFS <6 months (*n* = 22)	PFS ≥6 months (*n* = 16)	OR (95% CI) χ^2 test *p*-value
Brain metastases				
Yes	6 (15.8%)	6 (15.8%)	0 (0%)	
No	32 (84.2%)	16 (42.1%)	16 (42.1%)	0.500 (0.354–0.707) 0.023
Bone metastases				
Yes	13 (34.2%)	11 (28.9%)	2 (5.2%)	
No	25 (65.8%)	11 (28.9%)	14 (36.8%)	0.143 (0.026–0.783) *0.016*
Time from diagnosis to immunotherapy, months				
Median, months (median, range)	6.75 (0.7–4.9)	6.75 (0.7–4.9)	6.75 (2.1–4.0)	1.029 (0.972–1.089) *0.328*

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; IHC, immunohistochemistry; LAG-3, lymphocyte activation gene 3; NY-ESO-1, New York esophageal squamous cell carcinoma 1; OR, odds ratio; PD-1, Programmed cell death protein 1; PD-L1, programmed death-ligand 1; PFS, progression free survival. *P*-value.

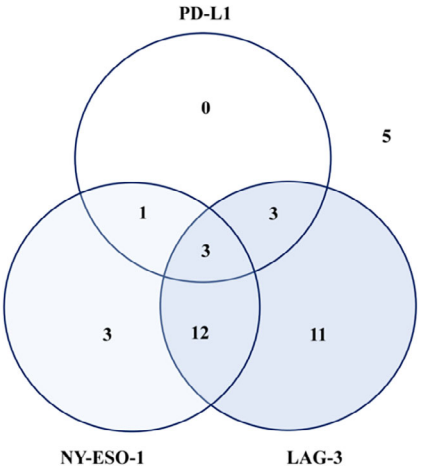

FIGURE 1 Tissue samples from 36 patients with NSCLC were evaluated for PD-L1, NY-ESO-1, and LAG-3 expression by IHC analysis. Three patients expressed all three proteins (triple-positive expression) and five had triple-negative expression. All patients expressing PD-L1 also co-expressed NY-ESO-1 or LAG-3

therapy (5/7, 71.4%); however, this relationship was not statistically significant. Conversely, liver (OR = 0.682, p = 0.014), brain (OR = 0.727, p = 0.030), and bone (OR = 4.0, p = 0.036) metastasis exerted negative effects on DCB, while other factors, such as histological type, number of previous lines of treatment, and PD-1 blockade type, did not significantly alter the DCB. In summary, single expression of either NY-ESO-1 or LAG-3 and double-positive LAG-3 and PD-L1 expression were the favorable independent factors related to DCB.

Progression-free survival

The median PFS of all patients was 5.5 months and clinical parameters were analyzed in relation to PFS, as shown in Table 2. Six parameters were linked with PFS: LAG-3 expression (hazard ratio [HR] = 0.198, p < 0.001); double-positive NY-ESO-1 and LAG-3 expression (HR = 0.295, p = 0.006); double-positive LAG-3 and PD-L1 expression (HR = 0.234, p = 0.050); liver metastasis (HR = 8.139, p < 0.0001); brain metastasis (HR = 3.822, p = 0.007); and bone metastasis (HR = 2.331, p = 0.035). No other factors were clinically significant. Despite using two different cutoff values for PD-L1 expression (5% and 50%), neither were statistically significant as a predictive factor for PFS (5% cutoff, HR = 0.566, 95% confidence interval [CI]: 0.224–1.428, p = 0.228; 50% cutoff, HR = 0.383, 95% CI: 0.113–1.293, p = 0.122). In addition, there was no statistically significant difference (p = 0.894) between the two anti-PD-1 treatments, nivolumab (4.5 months, 95% CI: 1.68–7.32) and pembrolizumab (5.6 months, 95% CI: 0.98–10.2). Multivariate analysis identified two factors with clinical value for PFS: LAG-3 and NY-ESO-1 co-expression correlated with a longer PFS (HR = 0.300, 95% CI: 0.109–0.823, p = 0.019), whereas liver metastasis at diagnosis (HR = 11.268, 95% CI: 3.091–41.075, p < 0.0001) was associated with a shorter PFS. We also analyzed PFS according to tumor tissue LAG-3, NY-ESO-1, and PD-L1 expression (Figure 2). Patients with PD-L1 expression (NR vs. 4.1, 95% CI: 1.6–6.5, p = 0.230) had a longer median PFS than those without PD-L1 expression. Similarly, patients with positive NY-ESO-1 expression had a longer PFS than those who did not express NY-ESO-1 (8.2 vs. 3.8, 95% CI: 4.5–11.8, p = 0.135), while patients expressing LAG-3 had a median PFS of 8.2 months (95% CI: 2.5–32.4) and those without LAG-3 expression had a PFS of 1.8 months (95% CI: 0.34–3.3; p < 0.0001). This indicated that the two factors—NY-ESO-1 and LAG-3—were statistically significant with respect to longer PFS.

Overall survival

The median OS of all patients was 12.5 months (range 1–42 months) and 24 patients had died at the cutoff date, with 68.1% (± 0.76%) of all patients alive at six months. Univariate analysis revealed that the significant prognostic factors for OS were similar to those related to a good PFS (Table 3). The patients expressing PD-L1 did not reach the median OS (p = 0.113), whereas those expressing LAG-3 and NY-ESO-1 reached 25 (95% CI: 6.5–43.5, p = 0.0058) and 27 (95% CI: 3.2–50.8, p = 0.081) months OS,

TABLE 2 Progression-free survival

		Univariate		Multivariate	
Variables	**N**	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age					
<65	17 (44.7%)	1			
≥65	21 (55.3%)	0.649 (0.303–1.389)	0.265	-	-
Sex					
Male	29 (76.3%)	1			
Female	9 (23.7%)	0.612 (0.231–1.620)	0.323	-	-
Smoking					
Never-smoker	11 (28.9%)	1			
Ever-smoker	27 (71.1%)	1.299 (0.549–3.077)	0.552	-	-
ECOG					
0	5 (13.2%)	1			
1	33 (86.8%)	2.601 (0.615–11.001)	0.194	-	-
Histology					
Squamous cell carcinoma	12 (31.6%)	1			
Nonsquamous cell carcinoma	26 (68.4%)	0.706 (0.322–1.545)	0.383	-	-
PD-L1 (5% cutoff)					
Negative	25 (65.8%)	1			
Positive	11 (28.9%)	0.566 (0.224–1.428)	0.228	-	-
PD-L1 (50% cutoff)					
Negative	29 (76.3%)	1			
Positive	7 (18.4%)	0.383 (0.113–1.293)	0.122	-	-
NY-ESO-1					
Negative	19 (50.0%)	1			
Positive	19 (50.0%)	0.504 (0.233–1.091)	0.082	-	-
LAG-3					
Negative	9 (23.7%)	1		1	
Positive	29 (76.3%)	0.198 (0.080–0.489)	<0.0001	0.399 (0.107–1.481)	0.170
NY-ESO-1 and LAG-3					
Negative	23 (60.5%)	1		1	
Positive	15 (39.5%)	0.295 (0.124–0.708)	0.006	0.300 (0.109–0.823)	0.019
NY-ESO-1 and PD-L1					
Negative	34 (89.5%)	1			
Positive	4 (10.5%)	0.526 (0.123–2.246)	0.386	-	-
LAG-3 and PD-L1					
Negative	32 (84.2%)	1		1	
Positive	6 (15.8%)	0.234 (0.055–0.997)	0.050	0.328 (0.072–1.488)	0.149
Previous lines of systemic treatment					
<2	29 (76.3%)	1			
≥2	9 (23.7%)	1.762 (0.769–4.040)	0.181	-	-
Liver metastases					
No	31 (81.6%)	1		1	
Yes	7 (18.4%)	8.139 (3.068–21.593)	<0.0001	11.268 (3.091–41.075)	<0.0001
Brain metastases					
No	32 (84.2%)	1		1	
Yes	6 (15.8%)	3.822 (1.433–10.195)	0.007	1.435 (0.339–6.078)	0.624
Bone metastases					

(Continues)

TABLE 2 (Continued)

Variables	N	Univariate		Multivariate	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
No	25 (65.8%)	1		1	
Yes	13 (34.2%)	2.331 (1.062–5.116)	0.035	1.241 (0.471–3.266)	0.662
Lung metastases					
No	16 (42.1%)				
Yes	22 (57.9%)	1.299 (0.602–2.804)	0.505	-	-

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; LAG-3, lymphocyte activation gene 3; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PD-L1, programmed death-ligand 1.

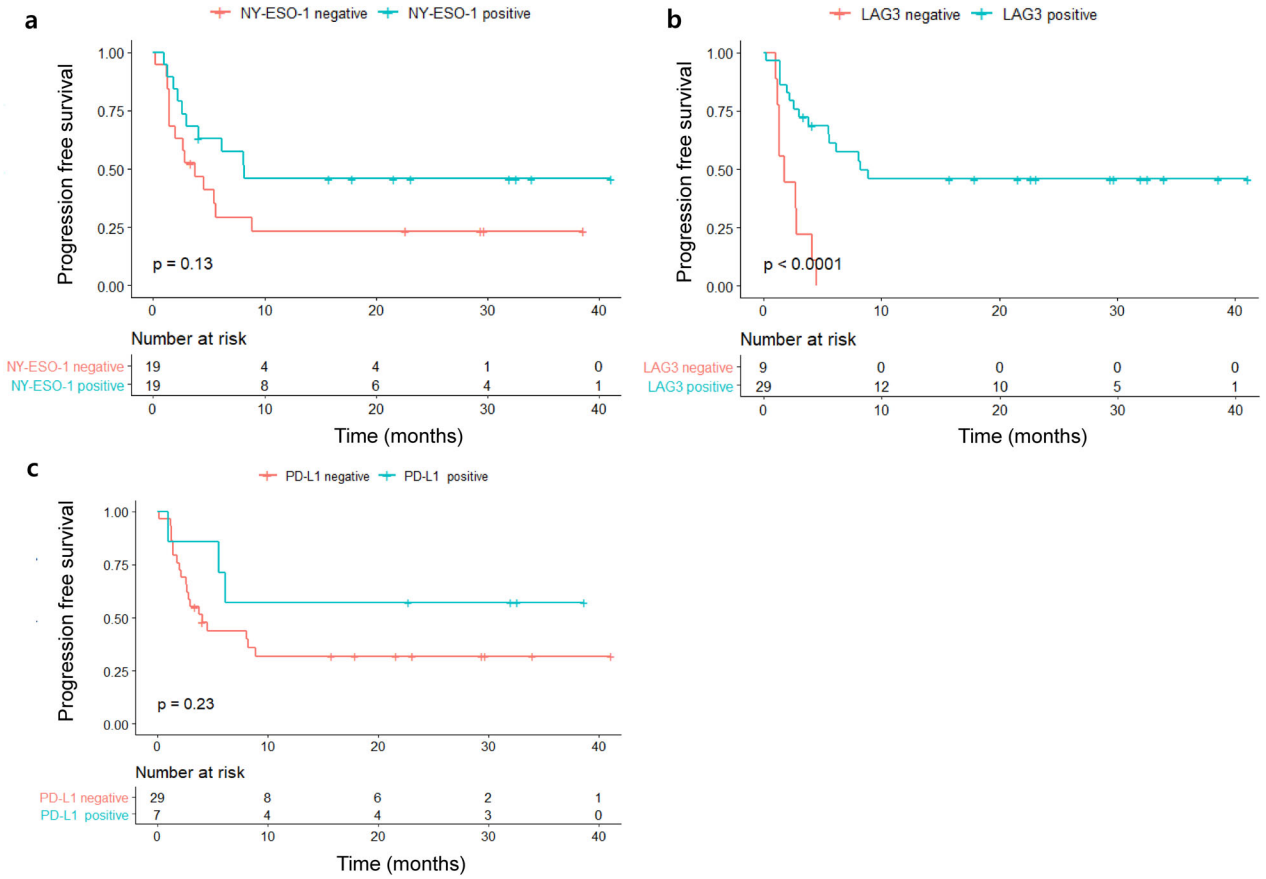

FIGURE 2 Progression-free survival (PFS) according to (a) NY-ESO-1, (b) LAG-3, and (c) PD-L1 expression. The expression of all three markers was associated with a longer PFS, but only LAG-3 was statistically significant

respectively (Figure 3). Moreover, the patients expressing LAG-3 and co-expressing NY-ESO-1 and LAG-3 showed a significantly higher OS of 16 (range 1–42) and 27 (range 5–42) months, respectively. Furthermore, patients with triple-positive expression displayed a median OS of 32 months (range 27–42, $n = 3$), whereas those with triple-negative expression had a median OS of seven months (range 2–13, $n = 5$). Multivariate analysis revealed that dual NY-ESO-1 and LAG-3 expression and liver metastasis at diagnosis were independent prognostic factors of OS, consistent with the PFS results.

Value of predictive biomarkers for clinical response to PD-1 antibody therapy

Finally, we estimated the negative predictive value (NPV) and positive predictive value (PPV) for the expression of NY-ESO-1 and LAG-3 in tumor cells (Table 4). NPV and PPV should be analyzed from large randomized clinical trials and should not be calculated from observational studies.[15] Although our study was small and retrospective, we calculated the PPV and NPV with respect to DCB to indicate clinical predictive importance and identify patients who

TABLE 3 Overall survival

Variables	N	Univariate		Multivariate	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age					
<65	17 (44.7%)	1			
≥65	21 (55.3%)	0.371(0.329–1.513)	0.706	-	-
Sex					
Male	29 (76.3%)	1			
Female	9 (23.7%)	0.538 (0.202–1.431)	0.214	-	-
Smoking					
Never-smoker	11 (28.9%)	1			
Ever-smoker	27 (71.1%)	1.429 (0.600–3.399)	0.420	-	-
ECOG					
0	5 (13.2%)	1			
1	33 (86.8%)	2.438 (0.585–10.534)	0.217	-	-
Histology					
Squamous cell carcinoma	12 (31.6%)	1			
Nonsquamous cell carcinoma	26 (68.4%)	0.614 (0.279–1.349)	0.224	-	-
PD-L1 (5% cutoff)					
Negative	25 (65.8%)	1			
Positive	11 (28.9%)	0.630 (0.250–1.586)	0.309	-	-
PD-L1 expression (50% cutoff)					
Negative	29 (76.3%)	1			
Positive	7 (18.4%)	0.394 (0.116–1.336)	0.135	-	-
NY-ESO-1					
Negative	19 (50.0%)	1			
Positive	19 (50.0%)	0.517 (0.239–1.121)	0.095	-	-
LAG-3					
Negative	9 (23.7%)	1		1	
Positive	29 (76.3%)	0.250 (0.140–0.599)	0.002	0.708 (0.237–2.118)	0.537
NY-ESO-1 and LAG-3					
Negative	23 (60.5%)	1		1	
Positive	15 (39.5%)	0.302 (0.126–0.721)	0.007	0.344 (0.126–0.939)	0.037
NY-ESO-1 and PD-L1					
Negative	34 (89.5%)	1			
Positive	4 (10.5%)	0.510 (0.120–2.174)	0.363	-	-
LAG-3 and PD-L1					
Negative	32 (84.2%)	1	0.053	1	
Positive	6 (15.8%)	0.238 (0.056–1.021)		0.332 (0.073–1.511)	0.154
Previous lines of systemic treatment					
<2	29 (76.3%)	1.519 (0.663–3.480)	0.323	-	-
≥2	9 (23.7%)				
Liver metastases					
No	31 (81.6%)	1		1	
Yes	7 (18.4%)	7.341 (2.623–20.540)	< 0.0001	4.897 (1.605–14.941)	0.005
Brain metastases					
No	32 (84.2%)	1		1	
Yes	6 (15.8%)	2.582 (1.006–6.624)	0.048	1.270 (0.366–4.404)	0.706
Bone metastases					

(Continues)

TABLE 3 (Continued)

Variables	N	Univariate		Multivariate	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
No	25 (65.8%)	1		1	
Yes	13 (34.2%)	2.210 (1.018–4.800)	0.045	1.839 (0.789–4.236)	0.153
Lung metastases					
No	16 (42.1%)				
Yes	22 (57.9%)	1.216 (0.561–2.635)	0.620	-	-

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; LAG-3, lymphocyte activation gene 3; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PD-L1, programmed death-ligand 1.

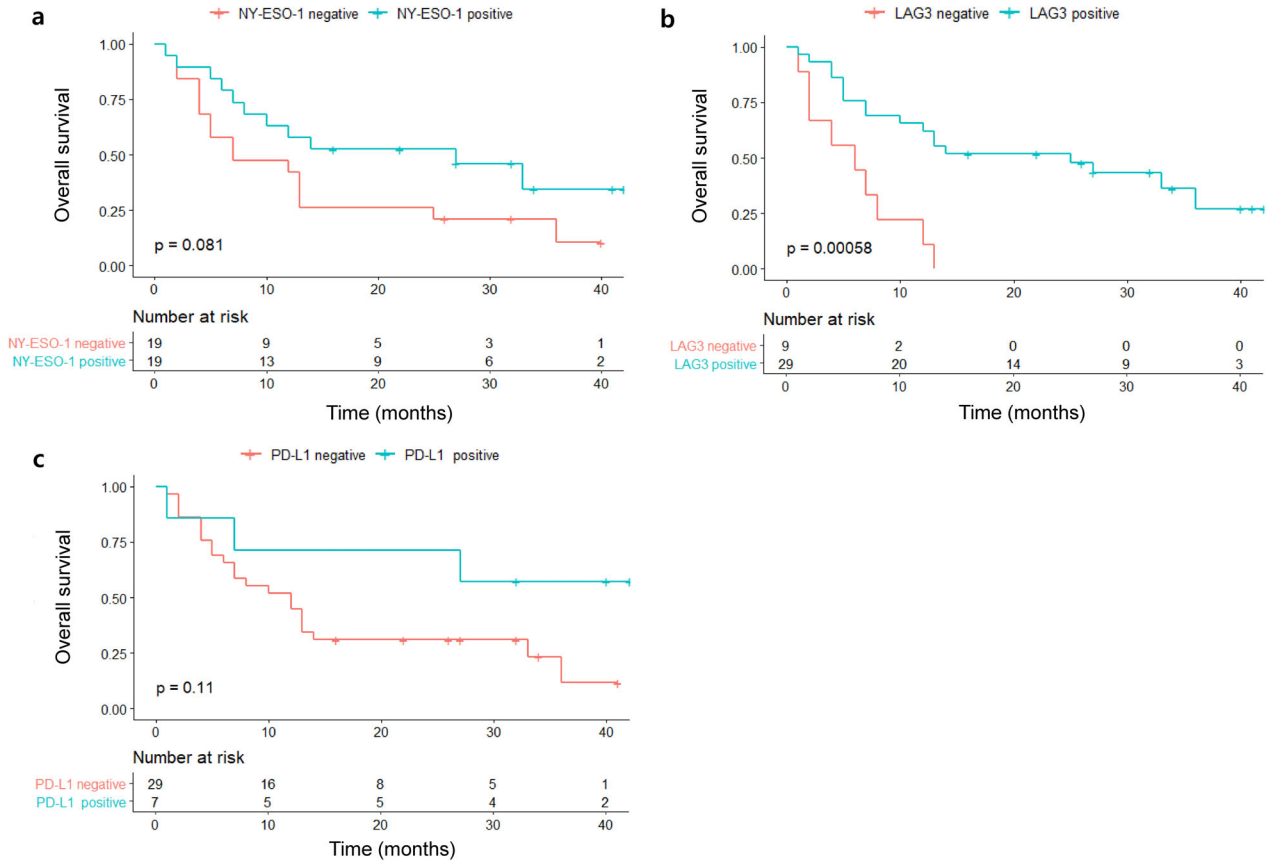

FIGURE 3 Overall survival (OS) according to (a) NY-ESO-1, (b) LAG-3, and (c) PD-L1 expression. LAG-3 positive patients displayed significantly longer OS than LAG-3-negative patients

TABLE 4 Positive (PPV) and negative (NPV) predictive value of the expression of each protein

	PPV	NPV
NY-ESO-1	57.89%	73.68%
LAG-3	55.17%	100%
PD-L1	71.43%	64.52%

Abbreviations: LAG-3, lymphocyte activation gene 3; NPV, negative predictive value; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PD-L1, programmed death-ligand 1; PPV, positive predictive value.

would respond well to anti PD-1 therapy. The significance of each measurement was determined using Fisher's exact test, revealing that the PPV of LAG-3, NY-ESO-1, and PD-L1 expression was 55.17%, 57.89%, and 71.43%, respectively, while the NPV of LAG-3 and NY-ESO-1 was 100% and 73.68%, respectively. We designed a decision classification tree model for the IHC results and DCB possibility using R version 3.4 (Figure 4). For predicting the survival outcome in our patients, LAG-3 and PD-L1 were selected, and PD-L1 IHC was not selected, as predictive markers to stratify

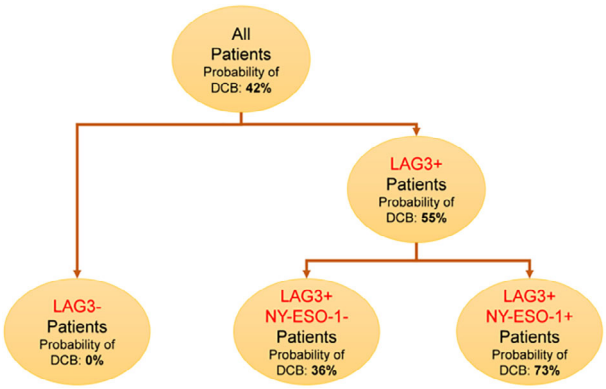

FIGURE 4 Decision tree for predicting durable clinical benefit (DCB) by expression of each marker. LAG-3, PD-L1, and NY-ESO-1 is root/intermediate node and leaf node was related to probability of survival. In this model, PD-L1 was excluded and only LAG-3 and NY-ESO-1 were included to stratify patients

patients. The probability of DCB in LAG-3+ and NY-ESO-1 + patient group was 73%, while that in patients without LAG-3 expression was 0%.

DISCUSSION

In this retrospective study, we explored the predictive value of NY-ESO-1 and LAG-3 expression in NSCLC patients treated with anti-PD-1 antibodies after failed platinum-based chemotherapy. We found that either NY-ESO-1 or LAG-3 expression was detected in all tumor samples from patients with high PD-L1 expression and was significantly associated with favorable patient outcomes, unlike PD-L1 expression. In addition, patients whose tumors expressed both NY-ESO-1 and LAG-3 had a high DCB rate and those with triple-positive PD-L1, LAG-3, and NY-ESO expression had a superior median OS and PFS to those with triple-negative expression. Furthermore, double LAG-3 and NY-ESO-1 expression was found to be a statistically independent predictor of both PFS and OS, while LAG-3 showed a good NPV.

Our results are consistent with previous studies that have reported synergy between LAG-3 and PD-1 in other tumor models, suggesting that dual immunotherapeutic efficacy may extend to multiple tumor subtypes.[16–20] Interestingly, the synergistic effects of PD-1 and LAG-3 were demonstrated using a murine model in which dual PD-1 and LAG-3 knockout reduced self-intolerance to induce tumor reduction, whereas single knockout exerted minimal immunopathological effects.[9] Anti-PD-1 monotherapy has only shown limited benefits in colon adenocarcinoma and fibrosarcoma tumor models, with a tumor response of 40% and 20%, respectively; however, 70% and 80% response rates have been reported following dual LAG-3 and PD-1 blockade, with similar results demonstrated in other cancer models including ovarian cancer, melanoma, and lymphoma.[9,21,22]

Likewise, NY-ESO-1 has been widely associated with a high objective response rate, long PFS/OS, and tumor reduction.[23] For instance, it has been suggested that NY-ESO-1-positive patients with NSCLC treated with PD-1/PD-L1 therapy display a good response, survival, and tumor reduction, regardless of tumor burden or CD8+ T-cell infiltration.[24] In this study, we found that NY-ESO-1-positive patients had a durable response for over six months alongside an improvement in PFS and OS that was not statistically significant. The reason for LAG-3 and NY-ESO-1 correlation with PFS and OS in patients using ICIs is attributed to the tumor microenvironment. LAG-3 enhances the negative regulation of T cells and represses CD 8+ effector T cells as a coinhibitory factor.[25] In the tumor microenvironment, LAG-3 is generally co-expressed with PD-1 and causes T cell exhaustion, thus reducing cytokine secretion[26,27] and producing favorable outcomes with ICI treatment. NY-ESO-1 displays restricted expression in normal tissue but becomes broadly expressed in malignancies and can elicit spontaneous T cell and humoral immune responses and induce strong immunogenicity during cancer treatment. The findings of this study confirm the roles of LAG-3 and NY-ESO-1 as surrogate markers for PD-1 blockade treatment and support these previous findings. Since our study was based on a limited number of patients with NSCLC treated with PD-1 blockade, additional studies are required to verify the predictive value of these markers in a larger NSCLC cohort alongside other molecules that affect aspects of the tumor microenvironment, such as T cell infiltration, tumor mutational burden, and microsatellite instability.

This study also demonstrated the role of PD-L1, NY-ESO-1, and LAG-3 by determining their PPV and NPV, observing a particularly high NPV for LAG-3; therefore, we suggest that LAG-3 and NY-ESO-1 could be used as surrogate markers for selecting patients that will show clinical benefits. We not only found that LAG-3 expression was significantly related with survival outcomes, but also that NY-ESO-1 and LAG-3 co-expression had clinical significance. For instance, patients with LAG-3 single expression showed a PFS and OS of 8.1 and 16.0 months, respectively, whereas those with LAG-3 and NY-ESO-1 co-expression displayed a PFS and OS of 15.8 and 27.0 months, respectively, which were longer that those of all patients. Thus, LAG-3 and NY-ESO-1 could be prognostic markers for PD-1 blockade treatment. Interestingly, the expression of LAG-3 and NY-ESO-1 in primary tumor cells was correlated with a longer OS than those with triple-negative expression of all three proteins, regardless of their PD-L1 expression. Although PD-L1 was first thought to be a promising biomarker for ICI sensitivity, the review of data from around 45 primary FDA ICI approval studies has since revealed that PD-L1 expression is only predictive in 28.9% of cases[3] and does not guarantee the response to PD-1/PD-L1 blockade treatment. Since patients with low PD-L1 expression also respond to PD-1 blockade treatment, high tumor PD-L1 expression cannot determine the response to anti-PD1 therapy and other methods are required to evaluate microenvironments with increased tumor T cell infiltration.[28] Consistently, we found that PD-L1 expression alone was insufficient to predict

treatment response and that the accuracy of the response prediction increased when various markers were measured together in a clinical setting. In particular, PD-L1 and LAG-3 co-expression had a high NPV, predicting poor treatment outcomes when patients negative for both PD-L1 and LAG-3 were treated with ICIs.

Despite these findings, our study has some limitations. First, our study population consisted of patients retrospectively selected from other clinical trials, and selection bias may have been introduced. The objective response rate (34.2%) observed in our study was higher than that of previously reported anti-PD1 therapy in NSCLC patients after platinum failure.[13,29–31] This was considered to be due to the small study population and the retrospective nature of our study. However, patients were treated by the coordinators of each clinical trial, meaning that the baseline characteristics of our cohort were uniform. Second, this study comprised a small number of patients and it was difficult to obtain tissue samples in some cases; therefore, further studies should be conducted with a larger sample size to verify the optimal predictive markers for PD-1 therapy. The heterogeneity of the tumor could be a confounding factor of NY-ESO-1 and LAG-3 expression in the small biopsy specimens in nine (23.7%) patients. However, recent studies of PD-L1, LAG-3 or NY-ESO-1 expression in NSCLC used small biopsy samples via core needle biopsy or bronchoscopy. Previous studies have suggested an excellent concordance of PD-L1 status comparison of evaluation performance on resection specimens and small biopsy specimens.[32,33] Hence, smaller samples appear adequate for assessment of PD-L1 expression, and we believe that it reflects real-world circumstances, and the more frequent incidence of patients undergoing bronchoscopy and needle biopsy.

In conclusion, patients with NSCLC who co-express NY-ESO-1 or LAG-3 with PD-L1 appear to exhibit greater clinical benefits and improved long-term survival following anti-PD-1 therapy. Moreover, NY-ESO-1 and LAG-3 could be considered as novel prognostic biomarkers, particularly LAG-3 as a negative prognostic marker, and the predictive value of these markers should be further evaluated in future clinical trials of NSCLC patients treated with ICIs.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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