



## ORIGINAL ARTICLE



# Serum Antibody Against NY-ESO-1 and XAGE1 Antigens Potentially Predicts Clinical Responses to Anti-Programmed Cell Death-1 Therapy in NSCLC

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## ABSTRACT

**Introduction:** Programmed cell death-1 (PD-1) inhibitors effectively treat NSCLC and prolong survival. Robust

biomarkers for predicting clinical benefits of good response and long survival with anti-PD-1 therapy have yet to be identified; therefore, predictive biomarkers are needed to select patients with benefits.

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Drs. Ohue and Kurose contributed equally to this work.

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**Methods:** We conducted a prospective study to explore whether serum antibody against NY-ESO-1 and/or XAGE1 cancer-testis antigens predicted primarily good clinical response and secondarily long survival with anti-PD-1 therapy for NSCLC. The serum antibody was detected by enzyme-linked immunosorbent assay, and tumor immune microenvironment and mutation burden were analyzed by immunohistochemistry and next-generation sequencing.

**Results:** In the discovery cohort ( $n = 13$ ), six antibody-positive NSCLC cases responded to anti-PD-1 therapy (two complete and four partial responses), whereas seven antibody-negative NSCLC cases did not. Antibody positivity was associated with good response and survival, regardless of tumor programmed death ligand 1 (PD-L1) expression, mutation burden, and CD8<sup>+</sup> T-cell infiltration. In the validation cohort ( $n = 75$ ), 17 antibody-positive NSCLC cases responded well to anti-PD-1 therapy as compared with 58 negative NSCLC cases (objective response rate 65% versus 19%,  $p = 0.0006$ ) and showed significantly prolonged progression-free survival and overall survival. Antibody titers highly correlated with tumor reduction rates. In the multivariate analysis, response biomarkers were tumor programmed death ligand 1 expression and antibody positivity, and only antibody positivity was a significantly better predictive biomarker of progression-free survival (hazard ratio = 0.4,  $p = 0.01$ ) and overall survival (hazard ratio = 0.2,  $p = 0.004$ ).

**Conclusions:** Our results suggest that NY-ESO-1 and/or XAGE1 serum antibodies are useful biomarkers for predicting clinical benefits in anti-PD-1 therapy for NSCLC and probably for other cancers.

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**Keywords:** Biomarker; Anti-programmed death 1 therapy; NSCLC; Cancer-testis antigen; Serum antibody

## Introduction

Lung cancer is the leading cause of death from cancer worldwide.<sup>1</sup> Most lung cancers, especially NSCLC, are diagnosed in the advanced stages and are resistant to conventional chemotherapy, resulting in poor prognosis. Recently, immunotherapy using immune-checkpoint inhibitors has prolonged NSCLC patient survival.<sup>2</sup> Programmed cell death-1 (PD-1) of an immune-checkpoint molecule is expressed on activated CD8<sup>+</sup> T cells and binds to programmed death ligand 1 (PD-L1) on tumor cells, resulting in T-cell exhaustion.<sup>3</sup> Therapeutic antibodies (Abs) for PD-1, nivolumab and pembrolizumab, inhibit this binding and reactivate CD8<sup>+</sup> T-cell with cytotoxic function

(cytotoxic T lymphocytes [CTLs]).<sup>3,4</sup> A number of clinical trials have since shown that anti-PD-1 therapy is effective in the treatment of various solid and hematologic malignancies including NSCLC. The response rate of NSCLC to anti-PD-1 monotherapy is approximately 20%; therefore, response biomarkers have been extensively investigated.<sup>4-8</sup> Although biomarkers such as tumor PD-L1 expression (tumor proportion score [TPS]), tumor mutation burden (TMB), microsatellite instability, T cell infiltration, and circulating PD-1<sup>+</sup>CD8<sup>+</sup> T cell are described, these markers are not so useful and convenient due to the reliability, cost, and time demands.<sup>4,5,7-9</sup>

Melanoma antigen-1 (MAGE-1, renamed MAGE-A1) of cancer-testis (CT) antigen was discovered as the first human tumor antigen, and hundreds of CT antigens have since been identified.<sup>10,11</sup> MAGE-A family members and New York esophageal squamous cell carcinoma-1 (NY-ESO-1) are broadly expressed in various human malignancies, and MAGE-A1, MAGE-A3, NY-ESO-1, SSX, and XAGE1 among CT antigens elicit spontaneous T cell and humoral immune responses in cancer patients. NY-ESO-1 has been extensively investigated as a target of cancer vaccines and T-cell therapy because it exhibits the highest immunogenicity among CT antigens.<sup>10-18</sup> XAGE1 is expressed in approximately 40% to 60% of lung adenocarcinomas, and the XAGE1 serum Ab is a good prognostic marker in advanced lung adenocarcinoma patients, as we reported previously.<sup>14,19,20</sup>

In cancer patients with serum Ab against NY-ESO-1 and XAGE1, the antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell are frequently detected in peripheral blood.<sup>12,14,15</sup> These findings suggest that NY-ESO-1 and XAGE1 are major immunodominant antigens and play important roles in the immune surveillance of NSCLC. The serum Ab positivity probably reflects the presence of activated antigen-specific T cell and PD-1/PD-L1-mediated immune suppression, suggesting good responses to anti-PD-1 therapy (Supplementary Fig. 1). Accordingly, we hypothesized that NY-ESO-1 and XAGE1 serum Abs have potential as response biomarkers in anti-PD-1 therapy for NSCLC, and conducted a prospective multicenter study to verify this hypothesis.

## Materials and Methods

### Study Design and Patients

This biomarker study was prospectively designed and performed to explore whether NY-ESO-1 and XAGE1 serum Abs predicted clinical responses and further patient survival with anti-PD-1 therapy, according to the Reporting Recommendations for Tumour Marker Prognostic Studies criteria as listed in the guideline.<sup>21</sup> This study was approved by the

Institutional Ethics Committee of Kawasaki Medical School (number 2071-7; clinical study registry, UMIN-CTR 000016678) and four medical centers in Japan, and was performed in accordance with the Declaration of Helsinki. For reference, we have investigated expression and immune responses and monitoring about CT antigens in human cancers including NSCLC for a long time.<sup>15,17,19,20,22-24</sup>

Patients who have advanced NSCLC with metastatic/recurrent and unresectable stages and postoperative recurrence were consecutively enrolled into this study from the above five medical centers between March 2016 and December 2018. Patient inclusion criteria were good performance status 0-2, good organ function, measurable lesions, and informed consent to this study. Exclusion criteria were active multiple primary malignancies and infections, autoimmune diseases, receiving intensive immunosuppressive agents, and pregnancy. Anti-PD-1 monotherapy with nivolumab or pembrolizumab was administered as a standard therapy for these NSCLC patients in a first-line (TPS  $\geq$  50% at diagnosis) or later setting according to government approval in Japan. Before anti-PD-1 therapy, NY-ESO-1 and XAGE1 serum Abs were measured using enzyme-linked immunosorbent assay (ELISA) by laboratory scientists.

Patients in the discovery cohort were stratified by their NY-ESO-1/XAGE1 serum Ab status for enrollment in this observational study and were analyzed. Then, when objective response rate (ORR) of anti-PD-1 monotherapy was 20% overall and at least 50% in the Ab-positive patients, and when the Ab-positive proportion was minimum 20% and maximum 25% in advanced NSCLC patients, the required sample size was 75 and 56 in the independent validation cohort, respectively. Finally, 75 patients with NSCLC were enrolled in the validation study. It was calculated in a priori power analysis for Fisher's exact test with the power level 0.8 and the significance level 0.05 by G\*Power calculator.<sup>25</sup> The reasons for estimated ORR 20% and 50% were based on the results of prior clinical trials and the ORR 50% in NSCLC of TPS greater than or equal to 50% treated with pembrolizumab, respectively, and the Ab-positive proportion 20% at minimum and 25% at maximum were referred to [Supplementary Table 1](#).<sup>2,5</sup> To assess the status of the Ab response, sera from patients were collected within 2 months before anti-PD-1 therapy, and Ab titers were measured. Clinical responses to anti-PD-1 therapy and the Ab status were double-blinded with each other by clinicians and laboratory scientists.

The primary and secondary endpoints in this study were the ORR to anti-PD-1 therapy, and progression-free survival (PFS) and overall survival (OS) after anti-PD-1

therapy, respectively, according to the NY-ESO-1/XAGE1 serum Ab status.

### Clinical Samples, Clinical Efficacy, and Survival Analysis

Patients had consented to Institutional Review Board-approved protocols permitting blood and tissue sample collection and sequencing. Tumor tissues and sera were obtained from all patients before anti-PD-1 therapy. Tumor tissues for whole-exome sequencing were freshly frozen material from only the discovery cohort patients before anti-PD-1 therapy. The *EGFR* mutation (*EGFR*mt) or *EML4-ALK*-fusion was identified by peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp and fluorescence in situ hybridization, respectively. As a negative control, sera from nonmalignant donors were independently obtained from previous study between 2015 and 2016.<sup>22</sup>

Target lesions at baseline were assessed according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), and the baseline sum of the longest diameters for target lesions was recorded and used to determine objective responses.<sup>26</sup> Initial responses to anti-PD-1 therapy were assessed by investigator-assessed RECIST v1.1 criteria, and a complete response (CR) and partial response (PR) were confirmed by a repeat imaging occurring at least 4 weeks after the initial identification of response.<sup>26</sup> Unconfirmed responses were considered stable disease (SD) or progressive disease (PD) dependent on the results of the second chest x-ray or computed tomography scan. PD showed more than 20% increase in the sum of diameters of target lesions from baseline sum or the appearance of new lesions.<sup>26</sup> All images were investigator-assessed by blinded reviewers.

PFS rates were assessed according to RECIST v1.1 and PFS and OS were analyzed by the Kaplan-Meier method. Differences in PFS and OS between patient subgroups were analyzed using the Log-rank test, and *p* values less than 0.05 were considered to be significant. PFS and OS for living patients were censored at the date of last known contact. The dates of PFS and OS after anti-PD-1 therapy were updated as December 31, 2018.

### Antibody Responses to NY-ESO-1 and XAGE1

Ab responses to the NY-ESO-1 and XAGE1 proteins were examined by ELISA, as we reported previously.<sup>20,27</sup> The recombinant NY-ESO-1 and synthetic XAGE1 protein (GL Biochemistry, Shanghai, China) (1  $\mu$ g/mL) in a coating buffer was adsorbed onto 96-well ELISA plates (Nunc, Roskilde, Denmark) and incubated at 4°C overnight. Plates were washed with

phosphate-buffered saline and blocked with 5% fetal calf serum/phosphate-buffered saline (200  $\mu$ L/well) at 37°C for 1 hour. After washing, 100  $\mu$ L of serially diluted serum was added to each well and incubated at 4°C for 2 hours. After washing, alkaline phosphatase affinity-purified goat anti-human immunoglobulin G, Fc $\gamma$  fragment-specific (1:5000) (Jackson Immuno Research, West Grove, Pennsylvania) was added to the wells and the plates were incubated at 37°C for 1 hour. After washing and development (AttoPhos AP fluorescent substrate system, Promega, Madison, Wisconsin), absorbance was read at an excitation of 440/30 and emission of 560/40 with a gain of 50.

The cutoff value was based on the reactivity of negative control sera from non-malignant donors (n = 60) and was defined as follows: the 95% confidence interval (CI) upper limit optical density (OD) value of the negative control serum pool. The extrapolated titer of patient serum samples was defined as the minimal dilution factor for which an O.D. greater than the cutoff was examinable. The Ab response was defined as positive for serum with extrapolated titers exceeding or equal to 100 ( $\geq 100$ ). The cutoff value of greater than or equal to 100 for positive was determined by previous findings that NY-ESO-1/XAGE1-specific T cells were frequently detected from cancer patients with Ab titer greater than or equal to 100.<sup>15,28</sup>

### Immunohistochemistry for Immune Microenvironment and CT Antigens

The tumor PD-L1 and major histocompatibility (MHC)-class I expression, and CD8<sup>+</sup> T cell, CD20<sup>+</sup> B cell, and CD163<sup>+</sup> M2-macrophage infiltration in tumor tissues before anti-PD-1 therapy were analyzed by immunohistochemistry (IHC).<sup>22</sup> Details of analysis methods are fully provided in the [Supplementary Materials](#). Tumor NY-ESO-1 and XAGE1 antigen expression before anti-PD-1 therapy were also analyzed by IHC. Four-micrometer-thick sections were deparaffinized with xylene and ethanol. Antigen retrieval was performed by microwave heating in antigen retrieval buffer (10 mM citrate buffer, pH 6.0) with a pressure cooker for 10 minutes. After the inactivation of endogenous peroxidase with 0.3% H<sub>2</sub>O<sub>2</sub> for 15 and 5 minutes, respectively, specimens were pre-incubated with serum-free blocking solution (Nacalai Tesque and DakoCytomation, Kyoto, Japan) for NY-ESO-1 and XAGE1, respectively. After washing, an anti-NY-ESO-1 mouse monoclonal Ab (clone E978, 1:100, Invitrogen, Carlsbad, California) and USO 9-13 monoclonal Ab (2  $\mu$ g/mL) were added and incubated at 4°C and room temperature overnight for NY-ESO-1 and

XAGE1, respectively. After washing, sample slides were stained by the streptavidin-biotin complex (SimpleStain MAX-PO kit; Nichirei, Tokyo, Japan), followed by a reaction with 3, 3'-diaminobenzidine in H<sub>2</sub>O<sub>2</sub> and counterstained with hematoxylin solution.

### Whole-Exome Sequencing and Detection of Tumor Somatic Mutations

DNA and RNA samples were prepared using AllPrep DNA/RNA/miRNA Universal Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was converted to DNA libraries for DNA sequencing using the SureSelect Target Enrichment System Capture Process (Agilent Technologies, Santa Clara, California). Details of analysis methods are fully provided in the [Supplementary Materials](#).

### RNA Sequencing and Immunogenomic Analysis of the Tumor Microenvironment

The characterization of the tumor immune microenvironment before anti-PD-1 therapy was further analyzed by next-generation sequencing. The expression of immune-related genes was extracted.<sup>29</sup> The enrichment of tumor-infiltrating lymphocytes was estimated by a single sample Gene Set Enrichment Analysis (ssGSEA) using gene sets provided by Charoentong et al.<sup>30-32</sup> Details of analysis methods are fully provided in the [Supplementary Materials](#).

### Statistical Analyses

Statistical analyses were performed with the two-sided Mann-Whitney U test for two groups using GraphPad Prism v.6 (Graphpad Prism Software, San Diego, California) and IBM SPSS Statistics 23 for Windows (IBM, New York, New York). In analyses of the relationship between each parameter, Spearman's correlation analysis was performed. PFS and OS were analyzed by the Kaplan-Meier method. Differences in PFS and OS between patient subgroups were analyzed using the Log-rank tests. To assess the relationship between a factor and PFS and OS, univariate and multivariate analyses were performed using Cox's proportional hazards regression model. We previously reported in lung adenocarcinoma that XAGE1 serum Ab was a good prognostic marker regardless of *EGFR* status and that XAGE1 and Galectin-9 were poor prognostic markers and tumor PD-L1 expression and T-cell infiltration were likely to be good prognostic ones.<sup>20,22</sup> These factors relevant to survival and the present study were investigated by a multivariate analysis. A multivariate analysis was performed by all factors using Cox's regression analysis (backwards stepwise model).

Results are expressed as the mean or 95% CI, and the threshold for significance was  $p$  less than 0.05.

## Results

### Relationship Between Antibody Responses to NY-ESO-1 and XAGE1 and Clinical Benefits of Good Response and Long Survival With Anti-PD-1 Therapy for NSCLC

In the discovery cohort, 13 patients with NSCLC who were treated with nivolumab were enrolled (Table 1), and the median follow-up time from the registration was 3.6 months (range, 0.5 to 24.0 months). These patients had previously been heavily treated with systemic chemotherapy. The CT antibody, serum Ab against the NY-ESO-1 and/or XAGE1 antigens, was positive in responders (CR and PR) and negative in nonresponders (SD and PD) (Fig. 1A). Two patients with CR had one each of NY-ESO-1 and XAGE1 Ab, whereas two of four patients with PR had NY-ESO-1 Ab and the remaining two had XAGE1 Ab. The CT antibody was not detected in

patients who were nonresponders, and there was a significant difference in the CT antibody titer between responders and nonresponders (Fig. 1A) ( $p < 0.01$ ). The best changes from the baseline of target lesions showed marked differences between CT antibody-positive and -negative patients (Fig. 1B). However, objective responses appeared to be associated with PD-L1 expression levels but not with the infiltration of various immune cells, cytokines, chemokines, or tumor MHC-class I expression levels analyzed by IHC and next-generation sequencing (Fig. 1B, Supplementary Fig. 2). CT antibody-positive patients obtained prolonged survival with anti-PD-1 therapy. Hazard ratios (HRs) of PFS and OS between CT antibody-positive and -negative patients were 0.17 (95% CI: 0.04–0.66) (Fig. 2A) and 0.15 (95% CI: 0.04–0.60) (Fig. 2B), respectively.

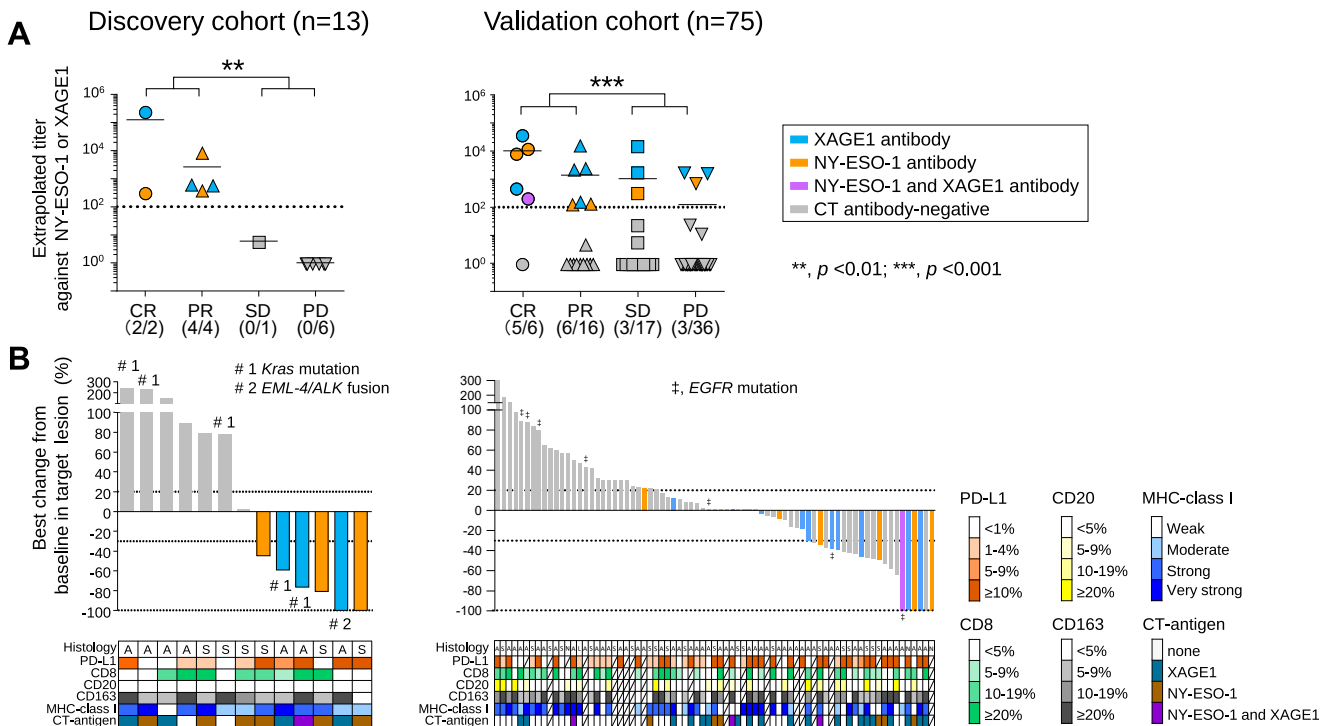
In the independent validation cohort, 75 patients with NSCLC were consecutively enrolled and received nivolumab or pembrolizumab (Table 1), and the median follow-up time from the registration was 7.3 months (range, 0.5 to 24.0 months). The CT antibody was positive in 17 of 75 (23%) patients (Table 1, Fig. 1A), comprising 10 XAGE1-positive Ab, 6 NY-ESO-1 Ab, and 1 with both Abs being positive (Fig. 1A). First-line anti-PD-1 therapy was given in 2 (12%) and 7 (12%) of 17 CT antibody-positive and 58 negative patients, respectively, and 66 of 75 (88%) patients received anti-PD-1 therapy after the second-line therapy, and 30 (40%) did after third-line therapy (Fig. 2C). Eleven of 17 (65%; 5 CRs and 6 PRs) CT antibody-positive patients responded to anti-PD-1 therapy, in contrast to 11 of 58 (19%; 1 CR and 10 PRs) CT antibody-negative patients (ORR 65% vs. 19%,  $p = 0.0006$ ), resulting in 29% (22 of 75) ORR in the validation cohort (Fig. 1A, Table 2). The sensitivity and specificity of CT antibody for response were 0.65 (95% CI: 0.38–0.86) and 0.81 (95% CI: 0.69–0.90), respectively. A significant difference was observed in the CT antibody titer between responders and nonresponders (Fig. 1A,  $p < 0.001$ ), and the greatest changes from the baseline of target lesions showed marked differences between CT antibody-positive and -negative patients (Fig. 1B). However, objective responses were not associated with CD8<sup>+</sup> T-cell infiltration or tumor PD-L1 and MHC-class I expression levels in appropriate available tissues before anti-PD-1 therapy (Fig. 1B). CT antibody-positive patients obtained prolonged survival with anti-PD-1 therapy; HRs of PFS and OS between CT antibody-positive and -negative patients were 0.42 (95% CI: 0.24–0.75) (Fig. 2A) and 0.21 (95% CI: 0.10–0.44) (Fig. 2B), respectively, even after the second line or the third line (Fig. 2C). In the multivariate analysis, response markers were smoking (odd ratio = 11, 95% CI: 1.0–113), high PD-L1 expression (odd ratio = 5.0, 95% CI: 1.0–26) and CT antibody positivity (odd ratio = 9.6, 95%

**Table 1. Patient Characteristics**

Characteristics	Discovery Cohort (n = 13)	Validation Cohort (n = 75)
Sex		
Male	12 (92)	55 (73)
Age, yr		
Mean (SD)	64 (7)	68 (10)
Race Asian	13 (100)	75 (100)
Smoking status		
Pack-years (SD)	77 (38)	41 (40)
Tumor type		
Squamous cell carcinoma	6 (46)	21 (28)
Adenocarcinoma	7 (54)	50 (67)
Others	0	4 (5)
Disease stage		
Metastatic/recurrent or unresectable	13 (100)	59 (79)
Postoperative recurrence	0	16 (21)
Brain metastasis		
Yes	4 (31)	20 (27)
Prior systemic regimens		
Median (range)	3 (1-8)	1 (0-9)
Driver-mutation status		
EGFR mutation	0	7 (9)
ALK translocation	1 (8)	0
Kras mutation	5 (38)	—
ROS1 translocation	0	—
Unknown	0	9 (12)
CT antibody		
Positive	6 (46)	17 (23)
Negative	7 (54)	58 (77)

Values shown are n (%) unless otherwise stated.

CT, cancer-testis antibody, serum antibody against NY-ESO-1 and/or XAGE1 antigen.



**Figure 1.** Relationship between antibody responses to NY-ESO-1 and XAGE1 cancer-testis (CT) antigens and clinical responses with anti-programmed death 1 (PD-1) therapy. **A**, Extrapolated CT antibody (serum antibody against NY-ESO-1 and/or XAGE1 CT antigen) titers in the discovery (*n* = 13) and validation (*n* = 75) cohorts according to clinical responses with anti-PD-1 therapy. Patient sera were collected within 2 months before anti-PD-1 therapy, and CT antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) and defined as positive for serum with an extrapolated titer exceeding or equal to 100 ( $\geq 100$ ). Clinical responses were assessed according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) guidelines. The numbers in parentheses indicate the number of CT antibody-positive patients/the number of patients with complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). (\**p* < 0.01; \*\*\**p* < 0.001 by two-sided Mann-Whitney U test.) **B**, Target lesions and their size at baseline were assessed according to RECIST v 1.1. Best change from baseline in the sum of the longest diameters of target lesions in patients who had greater than or equal to one evaluable post-baseline tumor assessment. Horizontal dotted-line represents the 20% threshold for declaring progression of target lesions and the -30% threshold for declaring response per RECIST v1.1. In the discovery and validation cohorts, all patients with NSCLC were treated with nivolumab or pembrolizumab, and 6 of 13 NSCLC and 21 of 75 NSCLC cases, respectively, in these cohorts were squamous cell carcinoma (S). Six patients in the discovery cohort had an *EML4-ALK* fusion or *Kras*-mutation tumor, and 7 in the validation cohort had an *EGFR*-mutation tumor. Programmed death ligand 1 (PD-L1) expression on tumor cells, CD8<sup>+</sup> T-cell, B-cell, and M2-macrophage infiltration, and major histocompatibility (MHC) class I and CT antigen expression levels in tumor tissues were evaluated by immunohistochemistry. A, adenocarcinoma; S, squamous cell carcinoma; L, large cell carcinoma.

CI: 1.6–57), and only CT antibody positivity was a significantly better predictive biomarker of PFS (HR = 0.4, 95% CI: 0.2–0.9) and OS (HR = 0.2, 95% CI: 0.1–0.8) after anti-PD-1 therapy (Table 2). Consequently, CT antibody-positive patients in the validation cohort also achieved clinical benefits of good response and long survival with anti-PD-1 therapy, even though they were heavily treated patients.

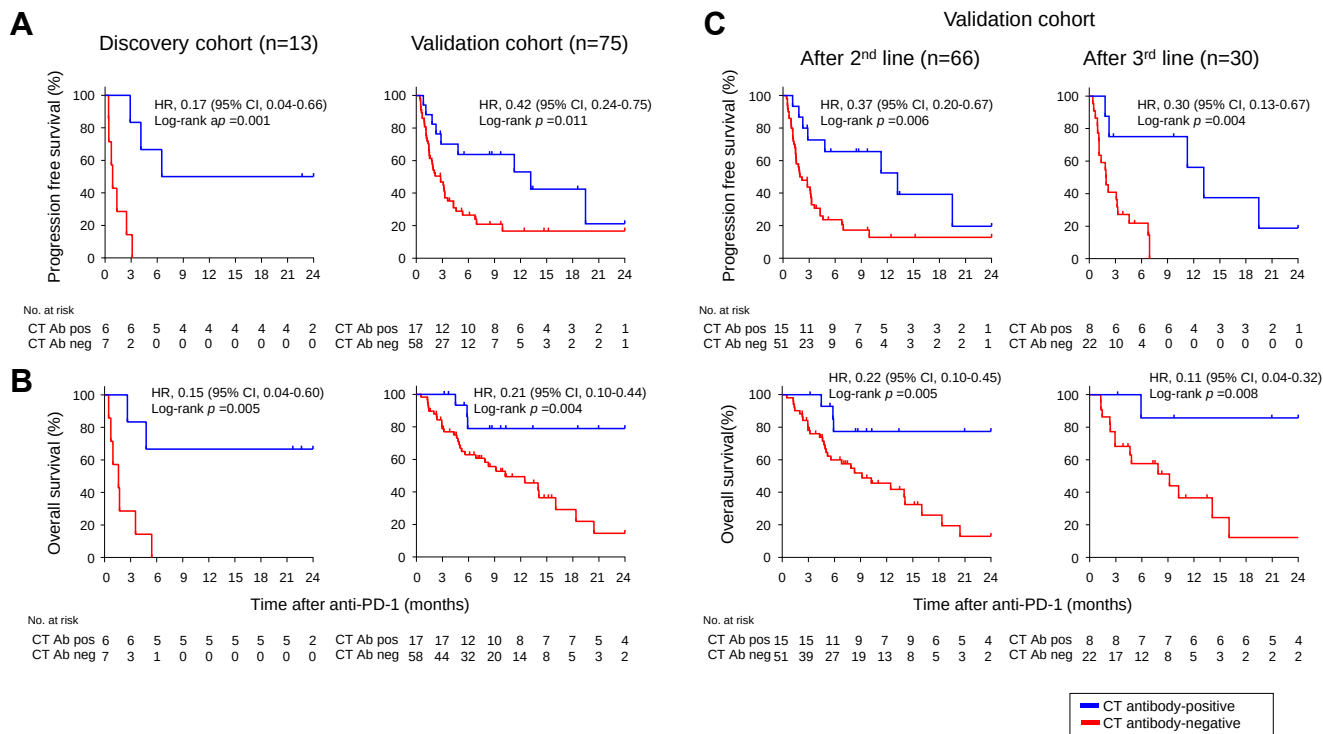
**Relationships Among CT Antigen Expression, CT Antibody, and Clinical Responses to Anti-PD-1 therapy**

We investigated the relationship between NY-ESO-1/XAGE1 expression levels and CT antibody titers in the discovery and validation cohorts. Figure 3A shows representative staining of tumor NY-ESO-1 and XAGE1

protein. No apparent correlations between NY-ESO-1/XAGE1 expression levels and CT antibody titers were observed (Fig. 3B). Then, we investigated the relationship between CT antibody titers before anti-PD-1 therapy and tumor reduction rates after anti-PD-1 therapy, resulting in a strong correlation between them (Fig. 3C) (*p* < 0.0001). This indicates that CT antibody is useful biomarker predicting responses to anti-PD-1 therapy.

**Relationships Among the CT Antibody, Tumor Missense-Mutation Burden (TMB), PD-L1 Expression, CD8 T-Cell Infiltration, and Clinical Benefits With Anti-PD-1 Therapy for NSCLC**

We evaluated TMB, tumor PD-L1 expression, CD8<sup>+</sup> T-cell, B-cell, and M2-macrophage infiltration, and tumor MHC-class I expression levels in the discovery cohort



**Figure 2.** Kaplan-Meier curves for the progression-free survival (PFS) and overall survival (OS) of patients according to cancer-testis (CT) antibody status in the discovery and validation cohorts after anti-programmed death 1 (PD-1) therapy. **A**, Kaplan-Meier curves for the PFS of patients according to the CT antibody (serum antibody against NY-ESO-1 and/or XAGE1 CT antigen) status in the discovery and validation cohorts after anti-PD-1 therapy. PFS was calculated from the date of anti-PD-1 therapy to the date of disease progression or death from any cause. Hazard ratio (HR) for PFS of CT antibody-positive patients, as compared to that of -negative patients, was calculated with Cox regression analysis, and 95% confidence interval (CI) was shown. **B**, Kaplan-Meier curves for the OS of patients according to the CT antibody status in the discovery and validation cohorts after anti-PD-1 therapy. OS was calculated from the date of anti-PD-1 therapy to the date of death from any cause. HR for OS of CT antibody-positive patients, as compared to that of negative patients, was calculated with Cox regression analysis, and 95% CI was shown. **C**, Kaplan-Meier curves for the PFS and OS of patients according to the CT antibody status in the validation cohorts after the second line ( $n = 66$ ) and the third line ( $n = 30$ ) anti-PD-1 therapy. PFS and OS were calculated as described above. HR for PFS and OS of CT antibody-positive patients, as compared to those of -negative patients, was calculated with Cox regression analysis, and 95% CI was shown. Ab, antibody; pos, positive; neg, negative.

before anti-PD-1 therapy (Fig. 1B, Fig. 4A), and representative staining was shown in Supplementary Fig. 3. ORR with anti-PD-1 therapy appeared to be associated with a high CT antibody titer (100%), TMB (75%), and PD-L1 expression (83%) (Fig. 4B), but not with CD8<sup>+</sup> T-cell, B-cell, and M2-macrophage infiltration or tumor MHC-class I expression levels (Fig. 1B). PFS, OS, and ORR according to TMB and the PD-L1 expression status, and the combination of these two were analyzed, showing better PFS and ORR with high TMB and PD-L1 expression than with low TMB and PD-L1 expression (Supplementary Fig. 4).

We investigated whether the combination of the CT antibody and tumor PD-L1 expression or CD8<sup>+</sup> T-cell infiltration has potential as a biomarker for clinical responses with anti-PD-1 therapy in 72 available tumor tissues (Supplementary Fig. 5). Regardless of the PD-L1

expression status, CT antibody-positive patients had higher ORR and longer PFS and OS than CT antibody-negative patients. Furthermore, regardless of CD8<sup>+</sup> T-cell infiltration, the former had higher ORR than the latter. For example, a patient who had lung adenocarcinoma with XAGE1 Ab and *EML4/ALK*-fusion achieved CR with anti-PD-1 therapy, and the marked intratumor infiltration of lymphocytes was observed after anti-PD-1 therapy (Supplementary Fig. 6). These results indicate that the CT antibody, even alone, is an independently useful biomarker for predicting clinical benefits of good response and long survival with anti-PD-1 therapy.

## Discussion

The present study showed that NSCLC patients with NY-ESO-1 and/or XAGE1 Ab obtained the significant

**Table 2.** Objective Response Rate, Progression-Free Survival, and Overall Survival With Anti-Programmed Death 1 Therapy, According to Patient Characteristics and Immunological Status

Characteristics	No. of Patients With CR or PR/Total	CR or PR Rate (95% CI) %	Odds Ratio for CR or PR (95% CI)	Median PFS	<i>p</i> Value	Univariate Analysis for PFS HR (95% CI)	Multivariate Analysis for PFS HR (95% CI)	Median OS	<i>p</i> Value	Univariate Analysis for OS HR (95% CI)	Multivariate Analysis for OS HR (95% CI)
<b>Sex</b>											
Male	16/55	29 (18 to 43)	—	2.8	0.58	1.0 (0.6 to 1.9)	—	NR	0.56	2.1 (0.9 to 4.9)	—
Female	6/20	30 (12 to 54)		4.3				18.4			
<b>Age, yr</b>											
< 65	7/24	29 (13 to 51)	—	1.9	0.32	1.3 (0.7 to 2.3)	—	12.5	0.25	1.5 (0.8 to 3.0)	—
≥ 65	15/51	29 (17 to 44)		3.6				18.4			
<b>Smoking status</b>											
Current or former smoker	17/53	32 (20 to 46)	11 (1.0 to 113)	3.3	0.37	0.7 (0.4 to 1.3)	0.4 (0.2 to 0.8)	18.4	0.72	1.1 (0.6 to 2.4)	0.8 (0.3 to 2.1)
Never-smoker	5/22	23 (8 to 45)		2.8				14.1			
<b>Tumor type</b>											
Squamous cell carcinoma	6/21	29 (11 to 52)	—	2.8	0.97	1.3 (0.7 to 2.4)	—	8.3	0.13	1.8 (0.8 to 3.7)	—
Non-squamous cell carcinoma	16/54	30 (18 to 44)		3.2				16.0			
<b>Disease stage</b>											
Metastatic/recurrent or unresectable	18/59	31 (19 to 44)	4.1 (0.5 to 34)	2.8	0.72	1.2 (0.6 to 2.2)	0.8 (0.4 to 1.9)	14.0	0.84	1.1 (0.5 to 2.5)	0.5 (0.2 to 1.5)
Postoperative recurrence	4/16	25 (7 to 52)		3.3				14.1			
<b>Brain metastasis</b>											
Present	6/20	30 (12 to 54)	0.3 (0.04 to 1.6)	2.4	0.34	1.3 (0.7 to 2.3)	2.2 (1.0 to 4.6)	5.2	0.09	1.8 (0.9 to 3.7)	5.2 (2.0 to 13)
None	16/55	29 (18 to 43)		3.3				14.1			
<b>Regimen line</b>											
First or second line	15/45	33 (20 to 49)	—	3.3	0.35	0.8 (0.5 to 1.4)	—	14.0	0.91	1.0 (0.5 to 1.9)	—
Later line	7/30	23 (10 to 42)		2.7				14.1			
<b>Driver mutation status (EGFR)</b>											
Positive	2/7	29 (4 to 71)	6.3 (0.2 to 198)	4.6	0.50	1.3 (0.6 to 2.9)	0.7 (0.2 to 2.6)	NR	0.31	0.5 (0.2 to 1.8)	0.5 (0.1 to 3.1)
Negative	18/59	31 (19 to 44)		3.2				14			
<b>CD8<sup>+</sup> T-cell infiltration</b>											
High	9/28	32 (16 to 52)	—	2.3	0.67	1.1 (0.6 to 2.0)	—	14.0	0.72	0.9 (0.4 to 1.9)	—
Low	10/31	32 (17 to 51)		3.2				14.1			

(continued)



Table 2. Continued

Characteristics	No. of Patients With CR or PR/Total	CR or PR Rate (95% CI) %	Odds Ratio for CR or PR (95% CI)	Univariate Analysis for PFS		Multivariate Analysis for PFS		Univariate Analysis for OS		Multivariate Analysis for OS	
				Median PFS	p Value	HR (95% CI)	HR (95% CI)	Median OS	p Value	HR (95% CI)	HR (95% CI)
PD-L1 expression score											
≥2	13/32	41 (24 to 59)	5.0 (1.0 to 26)	3.2	0.27	0.7 (0.4 to 1.3)	0.7 (0.3 to 1.4)	16.0	0.65	0.8 (0.4 to 1.8)	0.6 (0.2 to 1.4)
<2	5/27	19 (6 to 38)	—	2.8	—	—	—	7.9	—	—	—
CT antigen expression											
Positive	15/30	50 (31 to 69)	—	5.3	0.02	0.5 (0.3 to 0.9)	—	20.4	0.17	0.6 (0.3 to 1.3)	—
Negative	5/32	16 (5 to 33)	—	2.1	—	—	—	9.2	—	—	—
CT antibody											
Positive	11/17	65 (38 to 86)	9.6 (1.6 to 57)	13.2	0.01	0.4 (0.2 to 0.9)	0.4 (0.2 to 0.9)	NR	0.004	0.2 (0.1 to 0.7)	0.2 (0.1 to 0.8)
Negative	11/58	19 (10 to 31)	—	2.8	—	—	—	10.2	—	—	—

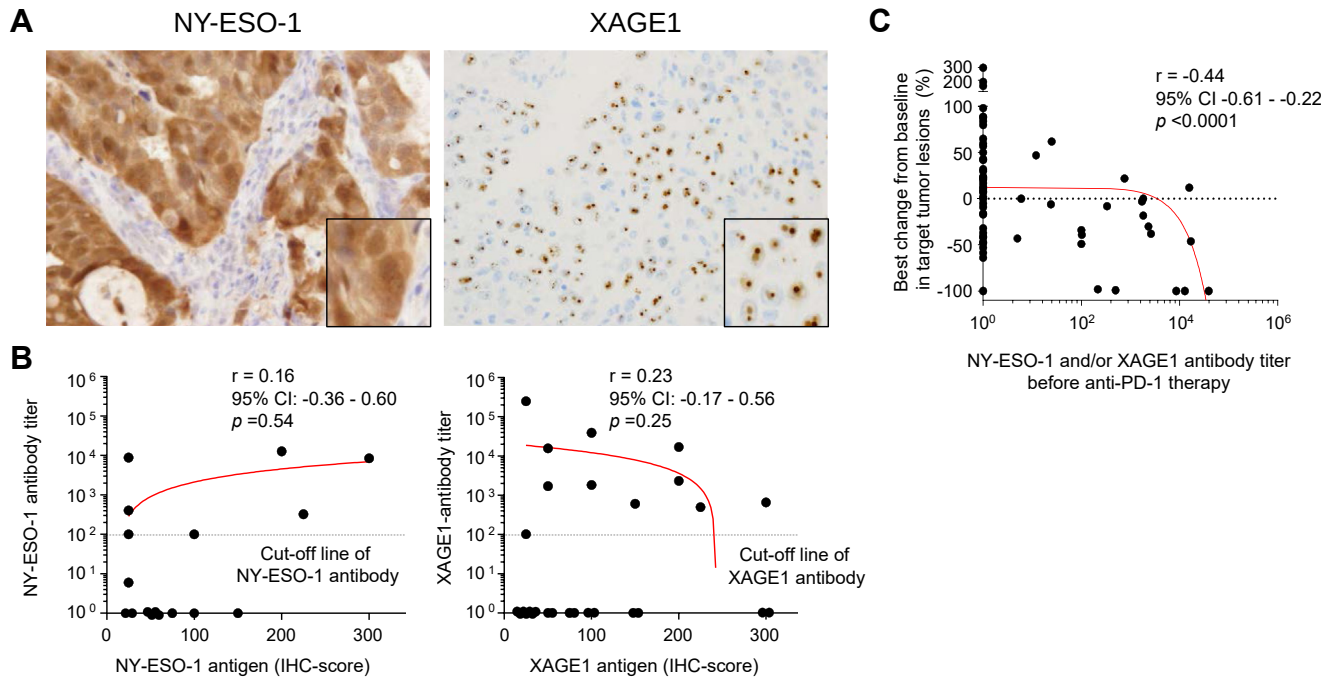
Multivariate analyses were performed using Logistic regression model to predict contributions of each variables to CR or PR probability. Univariate and multivariate analyses were performed using Cox's proportional hazards regression model to assess the relationship of all factors with PFS and OS. *p* < .05 was considered to be significant. CR, complete response; PR, partial response; CI, confidence interval; NR, not reached; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; PD-L1, programmed death ligand 1; CT, cancer testis antigen.

clinical benefits of high ORR and long PFS and OS with anti-PD-1 therapy, as compared with the Ab-negative patients. Additionally, Ab titers highly correlated with tumor reduction rates in anti-PD-1 therapy. Thus, our results suggest that strong immune surveillance was present in CT antibody-positive NSCLC patients who responded well to anti-PD-1 therapy, reversing immune suppression or tolerance. Therefore, NY-ESO-1 and XAGE1 Ab are considered to be convenient and useful biomarkers predicting clinical benefits of good response and long survival in anti-PD-1 therapy for NSCLC. Our novel findings in NSCLC would be extended to biomarker study in immunotherapies of other cancers because NY-ESO-1 is broadly expressed in many types of malignancy.

Here, we for the first time reported a strong correlation between NY-ESO-1 and XAGE1 Ab titers and tumor reduction rates with anti-PD-1 therapy for NSCLC. Similarly, Yuan et al.<sup>28</sup> reported a good correlation between NY-ESO-1 Ab titers and clinical responses and a close association between Ab-positivity with CD8<sup>+</sup> T-cell response and long survival, with anti-CTL antigen 4 (CTLA-4) therapy in melanoma. In addition, they addressed a close association between NY-ESO-1-specific Ab and CD8<sup>+</sup> T cell in patients who were responders. We also detected NY-ESO-1-specific CD8<sup>+</sup> T cell from peripheral blood of patient with CR after anti-PD-1 therapy (data not shown). Thus, it is speculated that CT antibody titers reflect cytotoxic activity levels of CT antigen-specific CD8<sup>+</sup> T cell.

In this study, many patients had been heavily treated with conventional chemotherapy; however, it is unlikely that CT antibody is associated with sensitivity to chemotherapy. Rather, CT antibody is considered to be a specific response biomarker for anti-PD-1 and -CTLA-4 therapy. There are few clinical reports focusing on CT antibody itself or the relationship between the antibody and cancer therapy, although Yuan et al.<sup>28</sup> showed good correlations between NY-ESO-1 Ab and clinical benefits with anti-CTLA-4 therapy in melanoma as described above. These results indicate the presence of activated CT antigen-specific CD8<sup>+</sup> T cell and checkpoint molecule-mediated strong immunosuppression in CT Ab-positive patients, reflecting good responses to checkpoint inhibitors even in patients heavily treated with chemotherapy. In the near future, these issues would be investigated in detail.

NY-ESO-1 and XAGE1 Ab were strongly associated with high response rates in anti-PD-1 therapy for NSCLC; however, the well-known biomarkers of tumor PD-L1 expression and TMB were also associated with better responses in the present study, as reported previously.<sup>4-8</sup> However, no relationship was noted between these Ab and the two biomarkers, indicating that these Ab are independent biomarkers in NSCLC. On the

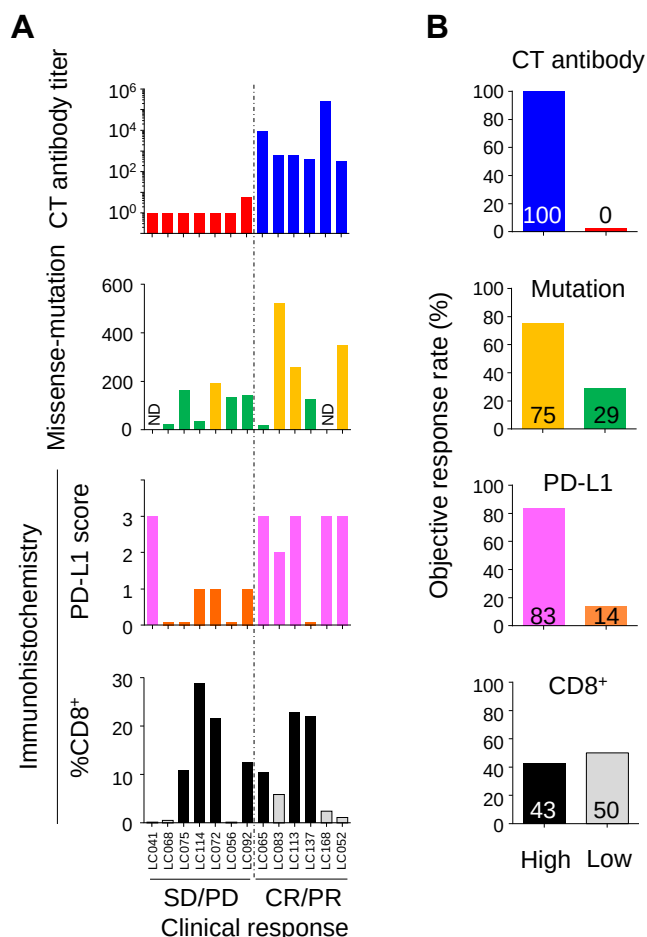


**Figure 3.** Relationships between cancer-testis (CT) antigen expression levels and the serum CT antibody titers, and between CT antibody titers and clinical responses with anti-programmed death 1 (PD-1) therapy. **A**, Typical immunostaining for NY-ESO-1 (left) and XAGE1 (right) protein in tumor cells. NY-ESO-1 and XAGE1 antigen are stained in the whole cells and nucleus, respectively. **B**, Relationship between NY-ESO-1/XAGE1 expression levels and the CT antibody (serum antibody against NY-ESO-1 and/or XAGE1 CT antigen) titers. The expression levels are represented as immunohistochemistry (IHC) score calculated by  $D$  (distribution)  $\times$   $I$  (intensity) in staining. Distribution ( $D$ ) is scored as 0, 0%; 25, 1 = 25%:50; 26~50%; 75, 51~75%; 100, 76~100%. Intensity ( $I$ ) is scored as 0, no signal; 1, weak; 2, moderate; 3, marked. **C**, Relationship between the CT antibody titers before anti-PD-1 therapy and tumor reduction rates after anti-PD-1 therapy was analyzed by using Spearman's correlation analysis. Best change from baseline in sum of the longest diameters of target lesions in patients who had greater than or equal to one evaluable post-baseline tumor assessment was calculated. Target lesions and their size were assessed according to Response Evaluation Criteria in Solid Tumors, version 1.1.

other hand, neither  $CD8^+$  T-cell, B-cell, and M2-macrophage infiltration nor tumor MHC-class I expression levels were associated with clinical responses. Many clinical trials on anti-PD-1 monotherapy for NSCLC have shown a correlation between tumor PD-L1 expression and ORR regardless of the antibodies used for PD-L1 staining, and ORRs were approximately 20%.<sup>4-6,8,33</sup> Notably, the ORR of 29% in our validation cohort ( $n = 75$ ) was similar to those in previous clinical trials on anti-PD-1 therapy, indicating that our results are not accidental and notable. Therefore, NY-ESO-1 and XAGE1 Ab need to be incorporated into stratification factors in future clinical trials on immune-checkpoint therapy for NSCLC.

NSCLC expresses multiple CT antigens such as NY-ESO-1, MAGE-A, SSSX, and GAGE family members at the protein or mRNA level, which may be related to tumor progression<sup>10-14,22</sup>; however, their functions are not fully known.<sup>10-12</sup> In the present study, NY-ESO-1 and XAGE1 protein expression was detected in 17 (23%) and 28 (37%) of 75 patients with available tumor tissues, respectively; however, CT antigen expression levels in biopsy specimens

did not correlate with CT antibody titers before anti-PD-1 therapy. Extensive clinicopathologic studies have shown that CT antigen expression in NSCLC is closely associated with the male sex, advanced diseases (stages III and IV), poor survival, and increased tumor burden.<sup>14</sup> Approximately 20% of NSCLC cases reportedly express NY-ESO-1 mRNA, which correlate with protein expression levels.<sup>34-36</sup> Among them, lung squamous cell carcinoma is reported to frequently express the NY-ESO-1 antigen with serum Ab in advanced stages, which is consistent with our results for squamous cell carcinoma.<sup>14</sup> NY-ESO-1-specific  $CD8^+$  T cell has been isolated from most patients with NY-ESO-1 Ab, and we also isolated NY-ESO-1-specific  $CD8^+$  T cell after anti-PD-1 therapy (data not shown).<sup>12,14</sup> Regarding XAGE1, approximately 40% to 60% of lung adenocarcinomas specifically express XAGE1, the Ab of which is frequently detected in advanced stages, as we reported previously.<sup>14,15,19,20,37</sup> Notably, NY-ESO-1 and XAGE1 among many CT antigens frequently elicit spontaneous  $CD4^+$  and  $CD8^+$  T cell and humoral immune responses in patients with advanced NSCLC, suggesting that these antigens are highly immunogenic and strongly related to the



**Figure 4.** Relationship between four predictive biomarkers and clinical responses with anti-programmed death 1 (PD-1) therapy. **A**, Relationships among the CT antibody (serum antibody against NY-ESO-1 and/or XAGE1 CT antigen), tumor missense-mutation burden, programmed death ligand 1 (PD-L1) expression, CD8<sup>+</sup> T-cell infiltration, and clinical responses with anti-PD-1 therapy in the discovery cohort (n = 13). Clinical responses were assessed according to Response Evaluation Criteria in Solid Tumors, version 1.1 guidelines. **B**, Objective response rates were shown according to high and low levels of the above four factors. High levels of each factor were defined for titer greater than or equal to 100 for the CT antibody, greater than or equal to 178 (median value) nonsynonymous single nucleotide variants for mutations, greater than or equal to score 2 (mean value) for PD-L1 expression, and greater than or equal to 10% (mean value) for %CD8<sup>+</sup> T cell, and a low level was less than the high level values. CT antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells were frequently detected in patients with CT antibody titer greater than or equal to 100. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

immune surveillance of NSCLC.<sup>14,15,23,37</sup> Thus, NY-ESO-1 and XAGE1 immunity in NSCLC are promising targets for immune-checkpoint therapy reversing immune suppression.

Our NSCLC patients with NY-ESO-1 and XAGE1 Ab responded particularly well to anti-PD-1 therapy. One

explanation for this result is the high immunogenicity of the NY-ESO-1 and XAGE1 antigens in NSCLC, as described above. Another reason is that some NSCLC patients' T cells simultaneously recognize multiple tumor antigens including CT antigens, as previously reported, and that anti-PD-1 and -CTLA-4 therapy elicited neoantigen-specific CD8<sup>+</sup> T-cell responses in NSCLC and melanoma, respectively.<sup>13,14,38-40</sup> Consequently, many antigens are considered to elicit antigen-specific CD8<sup>+</sup> T-cell responses destroying tumor cells. However, all tumor antigens do not always elicit CD8<sup>+</sup> T-cell responses; a previous study reported that mutant p53 peptides elicited CD4<sup>+</sup> T cell and humoral, but not CD8<sup>+</sup> T-cell responses.<sup>41</sup> The immunogenicity of each tumor antigen expressed in NSCLC must be investigated in more detail for future immunotherapies.

Although this study only included 88 NSCLC patients, two of seven patients with *EGFR*mt and XAGE1 Ab achieved CR and PR with anti-PD-1 therapy, and only one lung adenocarcinoma with *EML4-ALK* fusion and XAGE1 Ab had CR (Supplementary Fig. 5). These results indicate that XAGE1 Ab-positive NSCLC with *EGFR*mt or *EML4-ALK*-fusion responds to anti-PD-1 therapy. Several clinical trials on anti-PD-1 therapy have shown that NSCLC with *EGFR*mt usually does not have the clinical benefits of a better response and survival with anti-PD-1 therapy.<sup>7,42</sup> This may be because these NSCLC patients were non- or light smokers with low TMB.<sup>39,43</sup> Accordingly, *EGFR*mt may be a poor-response biomarker in anti-PD-1 therapy for NSCLC, whereas NY-ESO-1 and XAGE1 Ab may be good-response markers because NY-ESO-1 and XAGE1 are highly immunogenic in NSCLC, as we described above. NY-ESO-1 and XAGE1, as well as driver mutations of *EGFR*mt and *EML4-ALK* fusion, are tumor progression factors; however, CT antigens are highly immunogenic, whereas driver mutations may not be in NSCLC patients. Thus, NSCLC with *EGFR*mt may be divided into two types: CT antibody-positive and -negative, and CT antibody-positive NSCLC with *EGFR*mt may obtain clinical benefits with anti-PD-1 therapy, as shown here. This issue must be clarified in clinical trials on NSCLC with *EGFR*mt as soon as possible.

The limitations of the present study were the small number of patients included, the short follow-up time after anti-PD-1 therapy, and many analyses of small biopsy specimens. Larger clinical studies are needed to confirm the usefulness of the CT antibody as a predictive biomarker of the clinical benefits of good response and long survival associated with anti-PD-1 therapy. Now we are planning a large-scale study of patients with NSCLC.

In conclusion, patients who have NSCLC with NY-ESO-1 and XAGE1 Abs obtained significant clinical benefits of good response and long survival with anti-PD-1 therapy, and Ab titers correlated well with tumor reduction rates after anti-PD-1 therapy. NY-ESO-1 and

XAGE1 Ab are predictive biomarkers of clinical benefits with anti-PD-1 therapy for NSCLC, and must be incorporated into future clinical trials on immune checkpoint inhibitors as stratification factors.

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## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at [www.jto.org](http://www.jto.org) and at <https://doi.org/10.1016/j.jtho.2019.08.008>.

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