Survival of Lung Adenocarcinoma Patients Predicted from Expression of PD-L1, Galectin-9, and XAGE1 (GAGED2a) on Tumor Cells and Tumor-Infiltrating T Cells

Yoshihiro Ohue¹, Koji Kurose¹, Ryoei Nozawa², Midori Isobe³, Yumi Nishio¹, Tomonori Tanaka³, Yoshinori Doki⁴, Takashi Hori⁵, Junya Fukuoka³, Mikio Oka¹, and Eiichi Nakayama⁶

Abstract

The immune status of tumors varies, and this may affect the overall survival (OS) of patients. We examined tumors from 120 patients with lung adenocarcinomas with a tissue microarray for T-cell infiltration and the expression of PD-L1 and Galectin-9 (both ligands for inhibitory receptors on T cells), and cancer/testis (CT) antigen XAGE1 (GAGED2a; a tumor antigen often found on lung tumors) expression, to determine their relevance to OS. Patients defined as pStage I–IIIA could be grouped, based on the expression profiles of PD-L1, Galectin-9, and XAGE1, into cluster A, who had prolonged survival, and cluster B, who had shorter survival. The difference in survival of the clusters was confirmed separately for pStage I and pStage II–IIIA patients. Cluster A patients who also had CD4 and CD8 T-cell infiltration showed even better survival, as expected. The findings were confirmed by examining an independent validation cohort of 68 pStage I lung adenocarcinoma patients. Our data showed that PD-L1 expression was a positive indicator, whereas Galectin-9 and XAGE1 expression was negative. In vitro analyses suggested that PD-L1 expression was upregulated by IFNγ secreted from activated T cells in the tumor and Galectin-9 expression was counteracting those T cells. Thus, use of these immune markers enables the creation of a discriminant function with which to classify tumors and predict survival. Cancer Immunol Res; 4(12); 1049–60. © 2016 AACR.

Introduction

The infiltration of immune cells into a tumor can be a prognostic indicator (1, 2). Thus, immune scores would be useful as an alternative means for staging tumors, instead of the conventional anatomically based staging using the tumor–node–metastasis (TNM) classification (3). The possible immune parameters that could be utilized for evaluating the immune status in the tumor are as diverse as the various immune cells, molecules, cytokines, and chemokines.

T-cell inhibitory receptors and their ligands, and their roles in the tumor immune response, have been extensively studied. CTLA-4 (4), PD-1 (5, 6), and TIM-3 (7, 8) have been identified on the CD4 and CD8 T-cell surface. Each of these proteins contributes to the dysfunction of effector T cells in the tumor microenvironment when bound by their ligands; CD80 (B7-1) or CD86 (B7-2) for CTLA-4 (9, 10), PD-L1 (B7-H1) or PD-L2 (B7-DC; refs. 11–13) for PD-1, and Galectin-9 for TIM-3 (8). Targeting CTLA-4 (14–17), PD-1 (18–21), or PD-L1 (22, 23) using blocking antibodies provides a marked therapeutic effect in melanoma, renal cell carcinoma, and non–small cell lung cancer (NSCLC; ref. 24), suggesting that T-cell inhibition via these molecules is a common mechanism in antitumor responses in various tumor types.

Some studies have investigated the relation of inhibitory ligand expression on tumor cells to clinical outcomes. A higher frequency of PD-L1–expressing tumor cells has been correlated with poorer patient survival in some studies (25, 26), but better survival in other studies (27, 28). In the latter case, a higher number of PD-L1–expressing tumor cells was correlated with a higher number of infiltrating CD8 T cells or FoxP3 T regulatory cells (Treg), suggesting the involvement of inflammation in the tumor (29, 30). The IFNγ secreted from T cells induces PD-L1 expression in tumor cells (11). Galectin-9 regulates T-cell function through its receptor TIM-3 on the T-cell surface (8).

XAGE1 (GAGED2a) is a cancer/testis (CT) antigen, a class of antigens that is expressed in various tumors, but only in the testes in normal adult tissues (31–33). The XAGE1 antigen is expressed in 30% to 40% of lung adenocarcinomas (34, 35). Expression of...
XAGE1 correlates with shortened overall survival (OS), suggesting its relation to malignancy (36–38). In advanced lung adenocarcinoma patients, approximately half of patients with antigen-positive tumors naturally produced antibodies to XAGE1 (35, 38). CD4 and CD8 T-cell responses are frequently elicited in antibody-positive patients (35, 38). Antibody-positive patients showed prolongation of OS (37, 38).

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).

Materials and Methods

Clinical samples

Surgically resected primary lung cancer specimens, including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others from 329 patients, and metastatic lung tumors from 32 patients in the Toyama University Hospital archives, were used for the preparation of a TMA in the same block. Of these, complete clinical information including sex, age, smoking habit, and survival time was available in 120 adenocarcinoma patients. An independent validation cohort included 68 lung tumors from 32 patients in the Toyama University Hospital archives.

In this study, we investigated tumor-infiltrating CD4 and CD8 T cells and the expression of PD-L1, Galectin-9, and XAGE1 in stage I to IIIA lung adenocarcinomas using a tissue microarray (TMA), to deduce their contribution to OS. Using these parameters in combination, we developed a survival discriminant function for lung adenocarcinoma patients. Finally, we showed the functional role of PD-1- and TIM-3–expressing T cells in tumor-infiltrating lymphocytes (TIL).
anti–Ki-67-FITC were used for staining T cells. Anti-CD274 (PD-L1)-Brilliant Violet 421 and anti–Galectin-9-APC were used for staining tumor cell lines. To determine viable cells, Fixable Viability Dye-eFluor 506 was used. Annexin V and Ki-67 staining was done for assessment of apoptotic and proliferating cells according to BD Pharmingen manufacturer's instructions for the PE Annexin V Apoptosis Detection Kit I and FITC Mouse Anti-Human Ki-67 Set, respectively. Intracellular staining (ICS) was done with anti–CD274 (PD-L1), anti–Galectin-9, and anti–Ki-67-FITC according to the manufacturer's instructions.

The cells were washed and stained with a cell-surface marker for 30 minutes on ice. In ICS, the cells were washed, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. After washing in Perm/Wash solution (BD Biosciences), the pelleted cells were stained (30 minutes at 4°C) for intracellular molecules. In each staining, an isotype-matched control Ab was used. Analysis was done by FACS Canto II (BD Biosciences).

Isolation of TILs

TILs were freshly isolated from lung cancer tissues using a Medimachine (BioLab), as described previously (41).

Establishment of XAGE1 antigen–specific CD8 T-cell clones

This was done as previously described (35, 38).

Afatinib treatment of tumor cells

An EGFR-mutated tumor cell line, PC-9, and control EGFR wild-type cell line, OUI-LC-SK, were treated with afatinib (BIBW2992; Selleck Chemicals), which is an irreversible EGFR/HER2 inhibitor, at a concentration of 10 nmol/L for 3 days. After treatment, Galectin-9 release from apoptotic cells was measured by ELISA.

Treatment of T cells by Galectin-9

Cloned CD8 T cells (1 × 10⁴) were incubated with Galectin-9 protein hG9NC (GalPharma Co., Ltd.) at various concentrations for 8 hours. For antibody blocking, LEAF Purified anti-human Galectin-9 antibody, LEAF Purified anti-human TIM-3 antibody, or LEAF Purified Mouse IgG1, κ Isotype Ctrl Antibody (2 μg/mL; Supplementary Table S4) was used. After incubation, the cells were stained with Annexin V according to the manufacturer's instructions by FACS Canto II (BD Biosciences).

Overall survival

OS was determined as the time from an initial pathological diagnosis to death from any cause or the last follow-up of a patient. Pathological diagnosis involved the examination of resected tumor samples after surgery. OS was analyzed by the Kaplan–Meier method. Differences in survival between patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed using a Cox proportional hazards regression model to assess the association of a factor with OS. A P value of less than 0.05 was considered statistically significant.

Statistical analysis

Student t test or one-way ANOVA was used for multiple groups of quantitative data with normal distributions. Quantitative data without a normal distribution were analyzed by nonparametric tests. For contingency tables, the Fisher exact test was used. Furthermore, linear discriminant function analysis was used to discriminate between the two groups, and the standardized coefficients are shown with the 95% confidence interval (95% CI) by the bootstrap. All statistical analyses were performed using IBM SPSS Statistics 23 for Windows (IBM).

Results

Expression analysis of PD-L1, Galectin-9, and XAGE1

In preliminary experiments, we examined the expression of PD-1, TIM-3, BTLA, and LAG-3 on CD4 and CD8 T cells in PBMCs and TILs obtained from 11 lung cancer patients. Enhanced expression of PD-1 and TIM-3, but not BTLA or LAG-3, was observed in TILs compared with PBMCs, suggesting the relevance of the PD-1 and TIM-3 pathways in the local tumor environment. Expression of the T-cell inhibitory receptor ligands PD-L1 and Galectin-9, and a
cancer/testis antigen, XAGE1, in lung cancer was analyzed using TMA including 194 adenocarcinomas, 112 squamous cell carcinomas, 12 small cell carcinomas, and 11 others, plus 32 metastatic lung tumors by IHC (representative results in Fig. 1). The staining of PD-L1 and Galectin-9 was evaluated on the cell membrane and in the cytoplasm separately. Expression of either PD-L1 or Galectin-9 was observed predominantly in the cytoplasm compared with the cell membrane (Supplementary Table S5). With adenocarcinoma, the frequencies of high PD-L1 and Galectin-9 expression in the cell membrane or cytoplasm were 49% and 31%, respectively. On the other hand, with squamous cell carcinoma, the frequencies were 32% and 16%, respectively.

XAGE1 stain was in the nucleus of tumor cells (Fig. 1). Positive (more than or equal to 5% staining of cells) and negative (less than 5% staining of cells) staining were defined. The frequencies of XAGE1 expression in adenocarcinoma, squamous cell carcinoma, small cell carcinoma, others, and metastatic lung tumors were 28%, 3.6%, 0%, 18%, and 3.1%, respectively, consistent with our previous results (refs. 34, 36; Supplementary Table S6).

Thus, in lung adenocarcinoma, expressions of PD-L1, Galectin-9, or XAGE1 were observed at a high frequency. Subsequent analysis was conducted mainly on lung cancer of this histologic type.

**Analysis of CD4 and CD8 T cells and FoxP3⁺ Tregs in lung adenocarcinomas**

CD4 and CD8 T cells and FoxP3⁺ Tregs in lung adenocarcinoma were analyzed using TMA by IHC with an automated scan scope system (Fig. 2A). The staining score was defined for each T cell in terms of the staining strength from 3 to 0 by different colors, and the number of CD4 T cells with the same score was calculated automatically. Positive cells were defined as cells with scores of 3 and 2. The percentage of infiltrating T cells was the number of positive cells/all nucleated cells in TMA × 100. B (bottom). The percentage of infiltrating CD4 T cells in all TMA samples and a trendline (a red line) with a cubic curve are shown. The dotted line indicates the cutoff value at the inflection point. Bars in A and B, 50 and 100 μm, respectively.

**Figure 2.** A, IHC staining of CD4, CD8, and FoxP3⁺ cells in the lung adenocarcinoma TMA from patients LK033 and LK077. FoxP3⁺ cells were not observed in patient LK033, but were observed in patient LK077. B, An example of automated scan scope analysis of CD4 T cells from patient LK196. Each T cell was scored in terms of the staining intensity from 3 to 0 by different colors, and the number of CD4 T cells with the same score was calculated automatically. Positive cells were defined as cells with scores of 3 and 2. The percentage of infiltrating T cells was the number of positive cells/all nucleated cells in TMA × 100. B (bottom). The percentage of infiltrating CD4 T cells in all TMA samples and a trendline (a red line) with a cubic curve are shown. The dotted line indicates the cutoff value at the inflection point. Bars in A and B, 50 and 100 μm, respectively.
PD-L1, Galectin-9, and XAGE1 were clustered. Cluster A, expected values on the patients’ OS shown in Fig. 3, expression profile of OS with multiple immune parameters in all TMA samples. Supplementary Table S7 shows cutoff values for each T-cell parameter.

Analysis of the OS of patients expressing PD-L1, Galectin-9, and XAGE1
The OS of the patients with lung adenocarcinomas expressing PD-L1, Galectin-9, and XAGE1 was plotted by the Kaplan–Meier method (Fig. 3). The survival of patients with high PD-L1 tumors tended to be prolonged compared with patients with low/–PD-L1 tumors, although this was not significant (P = 0.09). Survival of the patients with high Galectin-9 tumors tended to be shorter compared with patients with low/–Galectin-9 tumors (P = 0.35). Survival of patients with XAGE1-positive tumors was shorter compared with patients with XAGE1-negative tumors (P = 0.01), consistent with our previous results (36, 38).

Effect of T-cell infiltration on OS
The effect on OS of CD4 and CD8 T cells and of FoxP3+ Treg infiltration in the tumor was then investigated in patients with lung adenocarcinoma. As shown in Supplementary Fig. S1, CD4 T-cell infiltration tended to prolong the survival of the patients (P = 0.07), whereas CD8 T-cell or FoxP3+ Treg infiltration showed no effect. No significant enhancement of prolongation was observed on using the CD4/FoxP3+ or CD8/FoxP3+ value instead of the CD4 or CD8 T-cell value, respectively. The survival of patients with CD4 and CD8 T-cell infiltration–positive tumors was prolonged compared with that of patients with T-cell infiltration–negative tumors (P = 0.04; Fig. 3).

OS with multiple immune parameters
Based on the possible effect of each parameter using binarized values on the patients’ OS shown in Fig. 3, expression profiles of PD-L1, Galectin-9, and XAGE1 were clustered. Cluster A, expected to show prolonged survival, included XAGE1-negative and PD-L1 high irrespective of Galectin-9 expression (a and e in Fig. 4A), or PD-L1 low/– if Galectin-9 were also low (g). On the other hand, cluster B, expected to show poorer survival, included XAGE1-positive with either PD-L1 high (b and f) or low/– (d and h), or Galectin-9 high (d and f) or low/– (b and h). It also included XAGE1-negative if Galectin-9 were was high (c). The survival of each group, a to h, is shown in Fig. 4A, and that of clusters A and B is shown in Fig. 4B. Those clusters were defined as pStage I–IIIA patients. To exclude the possibility that the difference was caused by including different stages of patients, survival was examined separately for pStage I and pStage II–IIIA patients. The survival of cluster A was significantly prolonged compared with cluster B in patients of either stage (Fig. 4C).

Furthermore, to define the patient subgroup in cluster A with even better survival, CD4 and CD8 T-cell infiltrations were incorporated (Fig. 4D). As expected, the survival of cluster A with TIL was markedly prolonged.

An independent cohort of 68 pStage I lung adenocarcinoma patients was examined for validation. The OS rates of patients belonging to clusters A and B are shown by the Kaplan–Meier method (Fig. 4E). The results were essentially the same as those of the study cohort.

Discriminant analyses classifying clusters A and B were then performed. A discriminant function separating clusters A and B was deduced using actual continual IHC scores instead of high and low/– with PD-L1 and Galectin-9 expression, and positive and negative expression (i.e., 1 or 0) for XAGE1 expression. Standardized coefficient values of the PD-L1 score, Galectin-9 score, and XAGE1 expression in the discriminant function are shown in Supplementary Table S8. The PD-L1 score was positively associated. On the other hand, the Galectin-9 score and XAGE1 expression were negatively associated. It should be...
noted that the significance of the histology differentiation and pStage in discrimination was much lower compared with the three immune parameters when incorporated in the function. The fact that no significant difference was observed with the TNM classification or pStage between clusters A and B in the patients’ characteristics in Supplementary Table S1 is consistent with the results.

Discriminant scores for each patient in the study cohort and also in the validation cohort are shown in Supplementary Fig. S2. Thus, the OS was predictable by calculating the discriminant score for clusters A and B. The OS of the patients in predicted clusters A (discriminant score more than or equal to 0) and B (discriminant score less than 0) was replotted for the study (Supplementary Fig. S3A) and validation (Supplementary Fig. S3B) cohorts. The results were essentially the same as the actual OS, as shown in Fig. 4B and C for the study cohort, and in Fig. 4E for the validation cohort, respectively.

The significance of OS prediction by the discriminant function was confirmed by univariate and multivariate Cox regression analysis, as shown in Supplementary Table S9.

Expression of PD-1 and TIM-3 inhibitory receptors on CD4 and CD8 TILs

We investigated the expression of PD-1 and TIM-3 inhibitory receptors on CD4 and CD8 T cells obtained from lung adenocarcinoma specimens. As shown in Fig. 5A, much stronger expression of PD-1 and TIM-3 was observed on CD4 and CD8 T cells in TILs compared with PBMCs. An increase in the PD-1$^+$ TIM-3$^+$ population and appearance of PD-1$^+$ TIM-3$^-$ and minor PD-1$^-$ TIM-3$^-$ populations was noticed in CD4 or CD8 T cells in TILs as compared with PBMCs (Fig. 5B and C). PD-1$^+$ TIM-3$^+$ and PD-1$^-$ TIM-3$^-$ populations in CD4 or CD8 TILs retained CD27 expression and showed the elevation of Ki-67 compared with the background level observed in the PD-1$^+$ TIM-3$^+$ population (Fig. 5D). Elevated expression of Annexin V was observed in the PD-1$^+$ TIM-3$^+$ and PD-1$^+$ TIM-3$^-$ populations, suggesting that TIM-3$^-$ cells were terminally differentiated cells. In CD8 T cells, loss of CD27 was observed in the PD-1$^+$ TIM-3$^+$ population, further suggesting that PD-1$^+$ TIM-3$^+$ cells were transient cells progressing to terminally differentiated PD-1$^+$ TIM-3$^-$.

Correlation of PD-L1 and Galectin-9 expression, and T-cell infiltration in the tumor

Next, we investigated the correlation of PD-L1 and Galectin-9 expression and T-cell infiltration in the tumor. As shown in Supplementary Fig. S4A and S4B, correlated expression of PD-L1, Galectin-9, and CD3 was observed at the periphery of the tumor nest. Either PD-L1 or Galectin-9 expression was correlated with the number of PD-1$^+$ TIM-3$^+$ cells and PD-1$^+$ TIM-3$^-$ cells, but inversely correlated with the number of PD-1$^+$ Tim-3$^+$ cells.
Because PD-L1 was involved positively and Galectin-9 was involved negatively in OS (Figs. 3 and 4; Supplementary Table S8), the findings suggested that PD-L1 expression in the tumor cells was upregulated by PD-1 and TIM-3 in inflammatory cells, probably through IFNγ secreted from those cells (see below). On the other hand, Galectin-9 expression in the tumor cells was upregulated intrinsically to counteract infiltrating T cells (see below). Lower expressions of PD-L1 and Galectin-9 in the tumor cells with increasing numbers of TIM-3+ T cells suggested that the reduction was due to an increase in dysfunctional/apoptotic T cells.

**PD-L1 expression by IFNγ and T-cell apoptosis by Galectin-9**

We subsequently investigated the augmentation of PD-L1 expression on the surface of tumor cells by IFNγ and CD8 T-cell apoptosis induction by soluble Galectin-9 released from lung cancer cell lines.

Expression of PD-L1 and Galectin-9 in 9 lung cancer cell lines by FACS is shown in Fig. 6. PD-L1 and Galectin-9 were detected on the cell surface and intracellularly in most adenocarcinoma or squamous cell carcinoma cell lines. Among 9 cell lines, 5 cell lines, A549, OU-LC-ON, OU-LC-SK, Sq-1, and RERI-LC-AI, were positive for XAGE1 mRNA expression (Supplementary Fig. S5).
As shown in Fig. 7A and B and Supplementary Fig. S6, treatment of the cell lines with IFNγ augmented PD-L1 expression on the cell surface, but had only a modest effect on its intracellular expression. No augmentation effect of IFNγ was observed on Galectin-9 expression either on the cell surface or intracellularly. Galectin-9 release from the cell lines was then examined. Galectin-9 release was observed in the medium of EGFR-mutated PC-9, but not EGFR wild-type OU-LC-SK after afatinib (EGFR-TKI) treatment (Fig. 7C). Moreover, Galectin-9 was easily detectable in the malignant pleural effusion, but not the serum, in patients with lung cancer (Fig. 7D). An increase in serum Galectin-9 was observed in 4 of 10 patients after chemotherapy (Fig. 7E). The findings suggested that Galectin-9 could be released and was detectable following tumor cell death. On the other hand, PD-L1 release was not detectable in the medium of EGFR-mutated lung cancer cell lines after afatinib treatment, in malignant pleural effusion of the patients, or in patient serum after chemotherapy (data not shown). A low amount of intracellular PD-L1 expression by FACS analysis (Fig. 6) suggested that the amount of PD-L1 released from the cells was below the detection level.

Using an XAGE1-specific CD8 T-cell clone established in our laboratory (Fig. 7F), the effect of Galectin-9 on TIM-3 signaling was examined. As shown in Fig. 7G and H, the addition of Galectin-9 to the CD8 T-cell clone induced apoptosis, and the induction of apoptosis was inhibited by the addition of an anti-Galectin-9 or anti-TIM-3 antibody in the medium.

**Discussion**

In this study, discriminant analyses were performed to discriminate clusters A and B that were determined based on the binarized values of PD-L1, Galectin-9, and XAGE1. Cluster A showed long survival and cluster B showed short survival of lung adenocarcinoma patients. The discriminant function was deduced by the use of actual continual IHC score values for the expression of PD-L1 and Galectin-9, and the positive (1) and negative (0) expression of the CT antigen XAGE1 in the tumor cells. Standardized coefficients of the discriminant function were +0.66 for the PD-L1 score, −0.93 for the Galectin-9 score, −1.26 for XAGE1 expression for the prediction of clusters A and B (P < 0.0001; Supplementary Table S8). The significance of those standardized coefficients was confirmed by the bootstrap method. The accuracy of discrimination was confirmed by cross-validation (Supplementary Fig. S2). A study using a similar approach was reported that showed the sum of four covariates, each multiplied by their individual coefficients, predicted the survival of patients with skeletal metastases from breast cancer (42).

The findings indicated the positive involvement of the PD-L1 score and negative involvement of the Galectin-9 score and XAGE1 expression in the discriminant function for survival prediction. Positive involvement of the PD-L1 score in survival could be explained by the primary contributor for prolonging survival as the infiltrating T cells, while the PD-L1 expression was upregulated passively, probably by IFNγ secreted from those T cells. In this study, we showed that PD-L1 expression was augmented by the treatment of lung cancer cell lines with IFNγ. Thus, it is likely that the higher PD-L1 expression on the tumor cells resulted from the effect of IFNγ secreted from the higher number of infiltrating immune T cells in the tumor microenvironment. The findings that the level of PD-L1 expression was in parallel with CD4 and CD8 T-cell infiltration with activating PD-1 TIM-3 and PD-L1 TIM-3 phenotypes, but inversely correlated with cell infiltration with dysfunctional PD-1 TIM-3 phenotypes in our study, were not contradictory. Similar findings were reported by other investigators (43).

On the other hand, the negative involvement of the Galectin-9 score for survival suggested a detrimental effect of Galectin-9 expression in tumor cells on the patients. It should be noted that Galectin-9 is highly expressed in the cytoplasm of lung cancer cell lines and is released following tumor cell death. Galectin-9 was released in the medium of EGFR-mutated lung cancer cell lines following treatment with afatinib (EGFR-TKI), and also in malignant pleural effusion and serum after chemotherapy. In malignant pleural effusion, an increase in Galectin-9 was easily detectable. The findings suggested that a significant amount of Galectin-9 could be released locally or systemically in these patients. Moreover, *in vitro* studies using lung cancer specific T-cell lines indicated apoptosis induction of TIM-3–positive CD8 T-cell clones following interaction with Galectin-9 that was inhibited by the addition of anti–Galectin-9 or an anti–TIM-3 antibody.
Thus, the release of soluble Galectin-9 could negatively regulate the T-cell function or even induce apoptosis through TIM-3 inhibitory receptor molecules. The findings of the expression of TIM-3 on CD4 and CD8 T cells in TILs, but not in PBMCs, shown in this and other (43, 44) studies suggest the functional relevance of the TIM-3 ligand in the tumor microenvironment.
The negative effect of XAGE1 expression on survival of the lung adenocarcinoma patients in the present TMA analysis was consistent with our findings reported previously (36, 38). The survival of the patients with XAGE1-positive lung adenocarcinomas without an immune response (no antibody response) against the antigen was somewhat shorter compared with that of the patients with XAGE1-negative tumors (38). However, the survival of the patients with XAGE1-positive tumors was prolonged when they showed an XAGE antibody response. Thus, it is conceivable that XAGE1 expression contributed to tumor malignancy, but the immune response to the antigen overcame its effect. The contribution to the malignancy of the CT antigens was also shown in MAGE-A3, NY-ESO-1, and SSX2 (45, 46). The poorer survival of the patients with tumors expressing those CT antigens was sometimes obscured by the beneficial effect of the immune responses against those antigens, as described. Therefore, the effect of the expression of the antigens on survival should be examined along with the presence or absence of the immune responses. On the other hand, recent findings suggested that the anti-CT antigen immune response was a surrogate for the immune response against newly generated antigens in the tumor that actually contributed to the antitumor response to the antigen overcame its effect. The contribution to the antigen overcame its effect. The contribution to the immune responses against newly generated antigens in the tumor that actually contributed to the antitumor response was potentially involved, but the antitumor response being involved positively and negatively, respectively, in prolonging OS (see above) suggested that PD-L1 expression was upregulated by IFNγ secreted from activated TILs, and Galectin-9 expression was counteracting those TILs in the tumor. The inverse correlation of PD-L1 and Galectin-9 expression with PD-L1 TIM-3 cells suggested that it was because TIM-3-expressing cells were dysfunctional.

Clinical trials that block the PD-L1/PD-L1 and TIM-3/Galectin 9 pathways in patients with XAGE1-positive and -negative tumors provide an opportunity to further evaluate these biomarker clusters prospectively for their predictive value.

Disclosure of Potential Conflicts of Interest
E. Nakayama is the head of research development at Polestar Co., reports receiving a commercial research grant from Polestar Co., and is a consultant/advisory board member for Medinet Co. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Ohue, K. Kurose, M. Oka, E. Nakayama
Development of methodology: Y. Ohue, K. Kurose, T. Tanaka, M. Oka
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ohue, K. Kurose, T. Horii, J. Fukuoka, M. Oka, E. Nakayama
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ohue, K. Kurose, R. Nosawa, T. Horii, J. Fukuoka, M. Oka, E. Nakayama
Writing, review, and/or revision of the manuscript: Y. Ohue, J. Fukuoka, M. Oka, E. Nakayama
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Ohue, K. Kurose, M. Isobe, Y. Nishio, Y. Doki, M. Oka, E. Nakayama
Study supervision: M. Oka, E. Nakayama
Other (financial support): M. Oka

Acknowledgments
We thank Dr. Masao Nakata of the Department of Surgery, Kawasaki Medical School Hospital, Kurashiki, Okayama, Japan, for help obtaining surgical specimens. We also thank Ms. Junko Mizuuchi for preparation of the manuscript.

Grant Support
This study was performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-DIRECT), Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported in part by grants-in-aid for Research on Applying Health Technology (H24-applying general 006) from the Ministry of Health, Labour and Welfare, Japan, by JSPS KAKENHI (23591169, 25430161, 26830117, 15K09235, 16K21533, and 16K18463), by a grant from Takeda Science Foundation, by a grant from Medical Research Encouragement Prize of The Japan Medical Association, and by grants from Kawasaki Medical School and Kawasaki University of Medical Welfare.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 27, 2015; revised September 7, 2016; accepted September 21, 2016; published OnlineFirst October 31, 2016.

References


Survival Prediction in Patients with Lung Cancer


42. Fourcade J, Sun Z, Benallaloua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor...


Survival of Lung Adenocarcinoma Patients Predicted from Expression of PD-L1, Galectin-9, and XAGE1 (GAGED2a) on Tumor Cells and Tumor-Infiltrating T Cells

Yoshihiro Ohue, Koji Kurose, Ryohei Nozawa, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0266

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2016/10/29/2326-6066.CIR-15-0266.DC1

Cited articles
This article cites 49 articles, 22 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/4/12/1049.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/4/12/1049.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.