ORIGINAL ARTICLE

The Tumor Immune Microenvironment Is Associated With Recurrence in Early-Stage Lung Adenocarcinoma

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ABSTRACT

Introduction: Immune checkpoint inhibitors have recently been approved for the treatment of early-stage NSCLC in the perioperative setting on the basis of phase 3 trials. However, the characteristics of such patients who are susceptible to recurrence after adjuvant chemotherapy or who are likely to benefit from postoperative immunotherapy have remained unclear.

Methods: This biomarker study (WJOG12219LTR) was designed to evaluate cancer stem cell markers (CD44 and CD133), programmed death-ligand 1 (PD-L1) expression on tumor cells, CD8 expression on tumor-infiltrating lymphocytes, and tumor mutation burden in completely resected stage II to IIIA NSCLC with the use of archived DNA and tissue samples from the prospective WJOG4107 trial. Tumors were classified as inflamed or noninflamed on the basis of the PD-L1 tumor proportion score and $CD8^+$ tumor-infiltrating lymphocyte density. The association between each potential biomarker and relapse-free survival (RFS) during adjuvant chemotherapy was assessed by Kaplan-Meier analysis.

Results: A total of 117 patients were included in this study. The median RFS was not reached (95% confidence intervals [CI]: 22.4 mo–not reached; n = 39) and 23.7 months (95% CI: 14.5–43.6; n = 41) in patients with inflamed or noninflamed adenocarcinoma, respectively (log-rank $p = 0.02$, hazard ratio of 0.52 [95% CI: 0.29–0.93]). Analysis of the combination of tumor inflammation category and *TP53* mutation status revealed that inflamed tumors without *TP53* mutations were associated with the longest RFS.

Conclusions: PD-L1 expression on tumor cells, $CD8^+$ T cell infiltration, and *TP53* mutation status may help identify patients with early-stage NSCLC susceptible to recurrence after adjuvant chemotherapy.

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Keywords: Non–small cell lung cancer; Adjuvant chemotherapy; Cancer stem cell; Tumor immune microenvironment

Introduction

Up to 50% of individuals with NSCLC who undergo surgery with curative intent experience disease recurrence within 5 years.[1] Adjuvant chemotherapy for early-stage NSCLC is currently considered the standard of care and is associated with an approximately 5% survival benefit at 5 years, which has remained unchanged for several decades.[2,3] However, adjuvant immunotherapy has recently been introduced for such patients in the perioperative setting. Adjuvant atezolizumab after platinum-based chemotherapy in individuals who underwent resection of stage IB to IIIA NSCLC was found to confer a disease-free survival benefit compared with best supportive care.[4] Pembrolizumab has also been approved for adjuvant treatment after resection and platinum-based chemotherapy for stage IB to IIIA NSCLC.[5] Given this scenario, there is a need for biomarkers to identify patients most likely to benefit from adjuvant cytotoxic chemotherapy and immunotherapy. The Lung Adjuvant Cisplatin Evaluation Biomarker (LACE-Bio) collaborative group has performed several pooled analyses or validation studies of promising biomarkers with a large cohort of patients who participated in four pivotal adjuvant chemotherapy trials: International Adjuvant Lung Cancer Trial, Adjuvant Navelbine International Trialist Association, National Cancer Institute of Canada Clinical Trials Group JBR.10 trial, and Cancer and Leukemia Group B 9633.[6–9] However, as of now, biomarkers to predict the relative benefit of chemotherapy for the perioperative period in early-stage NSCLC have not been established, and the optimal role of the cytotoxic chemotherapy backbone in the adjuvant setting has remained to be clarified.[10]

One factor contributing to this lack of established biomarkers is the wide range of biological behavior, ranging from highly aggressive to indolent, that is apparent for NSCLC. Cancer stem cells (CSCs) have been identified in multiple cancer types and play important roles in tumor development, relapse, and metastasis. Cell surface markers such as CD44 and CD133 have been used for the identification of these cells.[11–13] CSCs are maintained in a manner dependent on the operation of cell-intrinsic signaling pathways and the effects of infiltrating immune cell populations in the tumor immune microenvironment (TME). Expression of programmed death-ligand 1 (PD-L1) on tumor cells and the characteristics of infiltrating immune cells have recently been associated with prognosis in early-stage NSCLC.[9,14] A better understanding of the interplay between CSCs and the TME and its role in tumor recurrence would be expected to shed light on the determinants of the biological behavior of NSCLC.

We hypothesized that the combination of CSC markers and immune characteristics might contribute to the identification of individuals with early-stage NSCLC who are at high risk of recurrence after undergoing complete resection. We have, therefore, now performed an exploratory study to evaluate the potential relation of CSC markers (CD44 and CD133) and immune characteristics to survival in NSCLC patients who received adjuvant chemotherapy after complete resection in a previously reported trial (WJOG4107).[15]

Materials and Methods

Patients and Sample Collection

The present study (WJOG12219LTR) was designed as a biomarker study to evaluate CSC markers (CD44 and CD133), PD-L1 expression on tumor cells, CD8 expression on tumor-infiltrating lymphocytes (TILs), and tumor mutation burden (TMB) in completely resected stage II to IIIA NSCLC with the use of archived DNA and tissue samples from the WJOG4107 trial (UMIN000001658). The design and results of WJOG4107, a randomized phase 2 study of adjuvant chemotherapy for stage II to IIIA NSCLC, have been described previously.[15] Patients who underwent complete resection of stage II to IIIA NSCLC as classified according to the TNM staging system version 6, who were aged 20 to 74 years, and who had an Eastern Cooperative Oncology Group performance status of 0 or 1 were eligible. The patients were randomly assigned to receive oral S-1 either alone or together with cisplatin. The primary end points of WJOG4107 were the relapse-free survival (RFS) rate at 2 years and the identification of molecules whose expression in the tumor was significantly associated with patient outcome. RFS was defined as the time from initiation of the adjuvant therapy to disease recurrence or death, and relapse was assessed by means of positron emission tomography and a computed tomography scan of the chest at 6, 12, 18, and 24 months after initiation of the protocol treatment and with a follow-up period of 5 years, as defined in the protocol.

Patient characteristics and survival outcomes were obtained from the WJOG4107 data set. DNA was extracted from macrodissected and formalin-fixed, paraffin-embedded tumor specimens collected in the WJOG4107 trial and was archived at a controlled temperature of −80°C until analysis. Tumor tissue was obtained at resection surgery and was pathologically confirmed as NSCLC. The present study (WJOG12219LTR) was conducted in compliance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects of the Japanese government, and it was approved by the ethics committee of each participating institution. Among the patients enrolled in the WJOG4107 study, those for whom stored DNA and tissue samples were available were eligible for WJOG12219LTR (Supplementary Fig. 1).

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tumor tissue (thickness, 4 μm) were subjected to immunohistochemistry (IHC) with monoclonal antibodies to CD44 (Ventana 790-4537 [Roche Diagnostics, Basel, Switzerland]), to CD133 (Abcam ab19898 [Abcam, Cambridge, United Kingdom]), to PD-L1 (clone 28-8, Abcam ab205921), and CD8 (clone C8/144B, Agilent [Agilent Technologies, Santa Clara, CA]) and with the use of an automated stainer (Agilent Autostainer Link 48 or Leica Bond-Max [Agilent Technologies]). The stained slides were evaluated by a board-certified pathologist who was blinded to clinical outcomes. Scoring of CD44 and CD133 was performed according to the semiquantitative H-score, which was calculated by multiplying the cytomembrane staining intensity (0 = no staining, 1+ = weak staining, 2+ = moderate staining, 3+ = strong staining) by the percentage of stained cells (0% to 100%).[16] PD-L1 immunostaining was optimized with human placenta and tonsil as positive controls, and the percentage of tumor cells with membranous staining at any intensity was determined as the PD-L1 tumor proportion score (TPS),[17,18] with overall PD-L1 positivity being defined with a cutoff of greater than or equal to 1% of tumor cells (<1% defined as negative). TILs were evaluated on the basis of staining for CD8, with their number being determined at an absolute magnification of 400× (0.20 mm^2 per field). At least one and a maximum of five scanned fields of tumor regions were randomly chosen for each TIL count, and the TIL density in each tumor was calculated by dividing the number of TILs by the sum of the areas (mm^2) of the viewed fields.[18] TILs were defined as cells positive for CD8 at any staining intensity.

Next-Generation Sequencing

A targeted DNA library comprising approximately 1.65 Mb across 409 genes for panel sequencing was constructed with the use of an Oncomine Tumor Mutation Load Assay (ThermoFisher Scientific, Waltham, MA). In brief, tissue DNA was subjected to multiplex polymerase chain reaction amplification with an Ion AmpliSeq Library Kit Plus (ThermoFisher Scientific), Ion Xpress Barcode Adapters (ThermoFisher Scientific) were ligated to the amplicons, and the latter were then purified with the use of Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The purified libraries were pooled and then sequenced with the use of an Ion Torrent S5 XL instrument and Ion 550 Chip Kit (ThermoFisher Scientific). DNA

sequencing data were accessed through the Torrent Suite version 5.12 program (ThermoFisher Scientific). Reads were aligned with the hg19 human reference genome, and variants were called with the use of Ion Reporter ver. 5.10 (Thermo Fisher Scientific). Raw variant calls were filtered with a phred quality score of less than 50 or a variant read depth of less than 10, and they were manually checked with the Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA). Germline mutations were excluded with the use of the Genome Aggregation Database (gnomAD) and the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB). The TMB score was computed by the workflow of Ion Reporter version 5.10 and with the use of the Oncomine Tumor Mutation Load w2.0 workflow (ThermoFisher Scientific).[19] Loss of copy number for tumor suppressor genes and a copy number greater than or equal to 8 for oncogenes were considered pathogenic copy number variations. Frameshift mutations and nonsense mutations for tumor suppressor genes were also considered pathogenic. Pathogenicity for missense mutations was determined by first selecting nonsynonymous mutations that were defined as a hotspot by Ion Reporter version 5.10, had a Functional Analysis Through Hidden Markov Models score of greater than or equal to 0.7, or were defined as pathogenic or likely pathogenic by ClinVar (https://www.ncbi.nlm.nih.gov/clinvar).

Statistical Analysis

Categorical and continuous variables were summarized descriptively as percentage and median values, respectively. Differences in continuous variables were assessed with the Wilcoxon ranked sum test and those in categorical variables with Fisher's exact test. The cutoff values for CD44 H-score, CD133 H-score, $CD8^+$ TIL density, and TMB were determined by log-rank maximization analysis,[20] with that for PD-L1 TPS being clinically predetermined as 1%. Differences in RFS curves constructed by the Kaplan-Meier method were assessed with the log-rank test, and the Cox proportional hazards regression model was adopted to determine hazard ratios (HRs). All p values are two-sided, and confidence intervals (CIs) are at the 95% level, with statistical significance being defined as a p value of <0.05. All statistical analysis was performed with Stata IC version 14.2 (StataCorp LP, College Station, TX) or GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

Results

Patient Characteristics

Of the 200 patients treated in the WJOG4107 study, a total of 117 individuals had DNA and tissue samples available and were included in the present (WJOG12219LTR) study (Supplementary Fig. 1). The characteristics of these 117 patients, 57 of whom received S-1 monotherapy and 60 of whom received cisplatin plus S-1, are presented in Table 1. Among the WJOG12219LTR study population, the 2-year RFS rate was 63.0% for the S-1 group and 56.7% for the cisplatin–S-1 group, with these values being similar to those for the corresponding groups of the WJOG4107 trial (65.6% and 58.1%, respectively).

CD44 and CD133 Expression and Immune Characteristics

IHC revealed that CD44 expression (H-score) in tumor samples was significantly higher for patients with a PD-L1 TPS of greater than or equal to 1% than for those with a value of less than 1% (40 versus 20, $p = 0.008$), whereas CD133 expression tended to be lower in the former group (20 versus 50, $p = 0.08$) (Supplementary Fig. 2*A*). In contrast, neither CD44 nor CD133 expression differed significantly between patients with a high or low number of $CD8^+$ TILs (Supplementary Fig. 2*B*). Whereas CD133 expression did not differ significantly between patients with a low or high TMB, CD44 expression tended to be higher in the TMB-low group (Supplementary Fig. 2*C*). CD44 expression was significantly lower for individuals with lung adenocarcinoma ($n = 80$) than for those with other histologic types ($n = 37$), with an H-score of 20 versus 120, respectively ($p < 0.001$), whereas CD133 expression did not differ significantly between the two histologic groups (Supplementary Fig. 2*D*). The frequency distributions for CD44 H-score and CD133 H-score and representative IHC staining patterns for each protein are illustrated in Supplementary Figure 3. Among the 117 NSCLC cases, those with an H-score of 300 constituted 2.6% (3/117) for CD44 and 1.7% (2/117) for CD133.

IHC Staining and Clinical Outcome

Given that the expression of the CSC marker CD44 differed according to histologic type, we focused on lung adenocarcinoma for subsequent analysis. We first evaluated the relation of CSC markers or immune characteristics to RFS in patients with adenocarcinoma. We determined the cutoff values for CD44 H-score, CD133 H-score, and $CD8^+$ TIL density to be between 10 and 20, between 30 and 40, and between 84.9 and 85.5/mm^2, respectively, with the use of log-rank maximization analysis, and the patients were divided into two groups on the basis of each of these cutoff values. RFS did not differ significantly between patients with high versus low CD44 expression (median of 24.4 mo [95% CI: 15.2–not reached] versus 37.4 mo [95% CI: 16.7–not reached], respectively; log-rank test $p = 0.76$; HR of 1.10, with a

Table 1. Characteristics of the Study Patients According to Treatment Group

Characteristics	All (N = 117)	S-1 (n = 57)	Cisplatin + S-1 (n = 60)
Median age (range),[a] y	62 (37-74)	62 (37-74)	62.5 (42-74)
Sex			
Male	88 (75.2)	42 (73.7)	46 (76.7)
Female	29 (24.8)	15 (26.3)	14 (23.3)
Performance status			
0	78 (66.7)	42 (73.7)	36 (60.0)
1	27 (23.1)	11 (19.3)	16 (26.7)
Unknown	12 (10.3)	4 (7.0)	8 (13.3)
Smoking status[b]			
Current or former	91 (77.8)	44 (77.2)	47 (78.3)
Never	26 (22.2)	13 (22.8)	13 (21.7)
P stage			
II	69 (60.0)	35 (61.4)	34 (56.7)
IIIA	48 (41.0)	22 (38.6)	26 (43.3)
Histologic type			
Adenocarcinoma	80 (68.4)	38 (66.7)	42 (70.0)
Sq or other	37 (31.6)	19 (33.3)	18 (30.0)
Surgery			
Lobectomy	117 (100.0)	57 (100.0)	60 (100.0)
Lymph node dissection			
ND0-1	7 (6.0)	3 (5.3)	4 (6.7)
ND2	110 (94.0)	54 (94.7)	56 (93.3)

With the exception of age, values are number (percentage). Percentages may not add up to 100 because of rounding.
[a]At the start of adjuvant chemotherapy.
[b]Current smokers, individuals who had smoked a cigarette within the previous year; former smokers, those who had smoked at least 100 cigarettes but had quit more than 1 year before diagnosis; never-smokers, those who had smoked less than 100 cigarettes.
Sq, squamous cell carcinoma.

95% CI: 0.62–1.94) (Fig. 1*A*). Similarly, median RFS was 24.4 months (95% CI: 19.4–not reached) for the CD133-high group and 35.8 months (95% CI: 15.2–not reached) for the CD133-low group (log-rank test $p = 0.63$; HR of 1.15, with a 95% CI: 0.65–2.03) (Fig. 1*B*). In contrast, patients with a PD-L1 TPS of greater than or equal to 1% tended to have a longer RFS relative to those with a PD-L1 TPS of less than 1% (median of 53.7 mo [95% CI: 16.7–not reached] versus 24.2 mo [95% CI: 14.5–47.5]; log-rank test $p = 0.09$; HR of 0.61, with a 95% CI: 0.35–1.08) (Fig. 1*C*). Similarly, patients with a high $CD8^+$ TIL density had a longer RFS than did those with a low $CD8^+$ TIL density (median of 43.6 mo [95% CI: 24.1–not reached] versus 15.4 mo [95% CI: 9.3–47.6]; log-rank test $p = 0.04$; HR of 0.52, with a 95% CI: 0.29–0.95) (Fig. 1*D*).

TME Classification on the Basis of PD-L1 Expression and $CD8^+$ TIL Density

The 80 patients with adenocarcinoma for whom both PD-L1 and CD8 expression data were available were stratified into four TME groups on the basis of cutoffs of 1% for PD-L1 TPS and 84.9 to 85.5/mm^2 for $CD8^+$ TIL density (Fig. 2*A*). We defined tumors with a PD-L1 TPS of greater than or equal to 1% and a high $CD8^+$ TIL density on the basis of this stratification as "inflamed" (n = 39) and all other tumors as "noninflamed" (n = 41). The characteristics of the patients with inflamed or non-inflamed tumors are presented in Supplementary Table 1. The median RFS was not reached (95% CI: 22.4 mo–not reached) in patients with inflamed tumors versus 23.7 months (95% CI: 14.5–43.6) in those with noninflamed tumors (log-rank test $p = 0.02$; HR of 0.52, with a 95% CI: 0.29–0.93) (Fig. 2*B*), suggesting that the combination of PD-L1 TPS and $CD8^+$ TIL density might serve as a biomarker of recurrence. An association was apparent between the CD44 H-score and tumor inflammation category (median of 20 versus 10 for inflamed versus noninflamed tumors, respectively, $p = 0.04$), whereas no such association was detected for the CD133 H-score (median of 20 versus 40, respectively, $p = 0.13$) (Fig. 2*C*).

Genomic Features and Clinical Outcome

We determined the cutoff value for TMB to be between 10.1 and 10.8/Mb by log-rank maximization analysis. RFS did not differ significantly between adenocarcinoma patients with a high versus low TMB (median of not reached [95% CI: 14.5 mo–not reached] versus 34.0 mo [95% CI: 16.7–not reached], respectively;

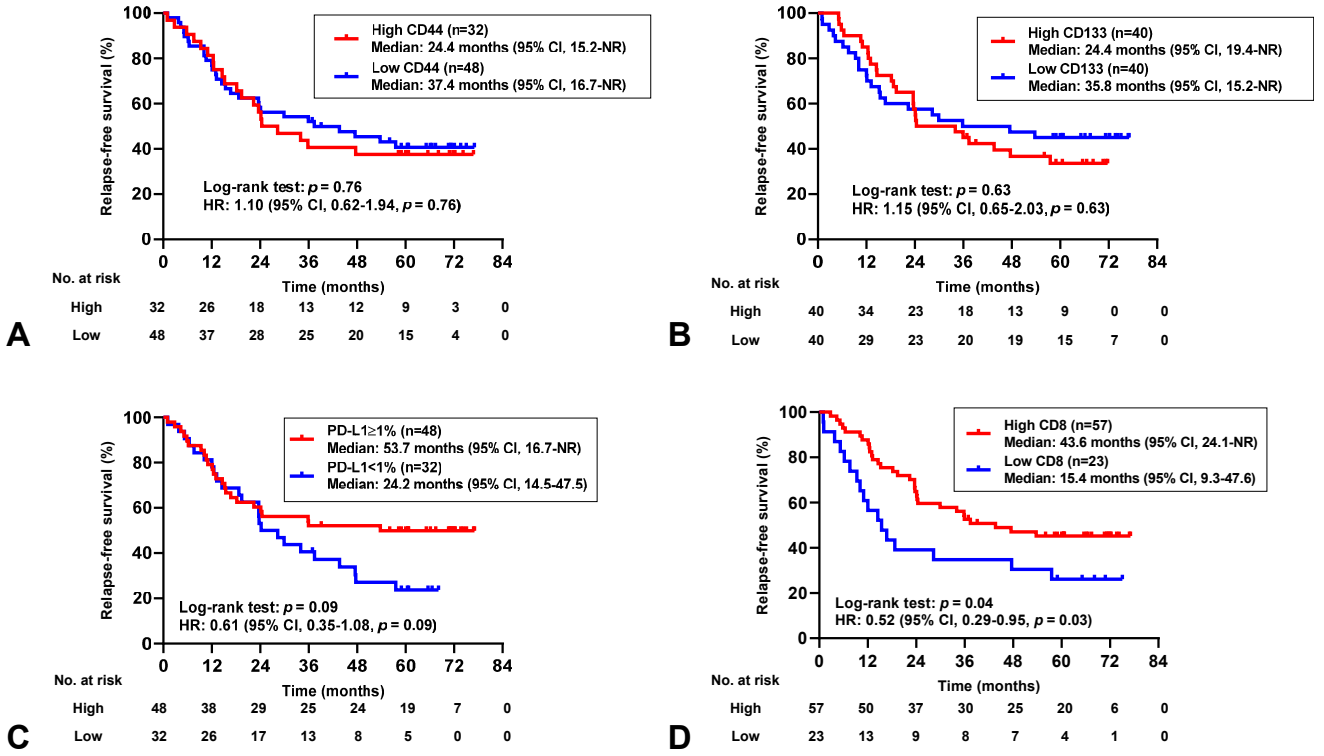

Figure 1. Kaplan-Meier curves for relapse-free survival of study patients with lung adenocarcinoma according to the CD44 H-score (*A*), CD133 H-score (*B*), PD-L1 tumor proportion score (*C*), or $CD8^+$ tumor-infiltrating lymphocyte density (*D*) as determined by immunohistochemistry of tumor samples. The cutoff for PD-L1 was 1%, and those for CD44 (H-score 10-20), CD133 (H-score 30-40), and CD8 (84.9-85.5 cells/mm^2) were determined by log-rank maximization analysis. CI, confidence interval; NR, not reached; HR, hazard ratio; PD-L1, programmed death-ligand 1.

log-rank test $p = 0.15$; HR of 0.57, with a 95% CI: 0.25–1.29) (Fig. 3*A*). Among the 409 genes evaluated, the two with the highest frequency of genetic alterations, *TP53* and *EGFR* (Supplementary Fig. 4), were investigated further. Such investigation also revealed no significant difference in RFS between individuals positive or negative for *EGFR* mutations (median of 34.0 mo [95% CI: 16.7–not reached] versus 35.9 mo [95% CI: 15.4–not reached], respectively; log-rank test $p = 0.71$; HR of 1.13, with a 95% CI: 0.60–2.13) (Fig. 3*B*). RFS tended to be shorter in patients with *TP53* mutations than in those without such mutations (median of 23.7 mo [95% CI: 13.0–47.6] versus not reached [95% CI: 28.3 mo–not reached], respectively; log-rank test $p = 0.05$; HR of 1.91, with a 95% CI: 0.98–3.73) (Fig. 3*C*).

Neither *EGFR* nor *TP53* mutations were associated with the tumor inflammation category (Table 2). Finally, we evaluated the clinical utility of the combination of tumor inflammation category and *TP53* mutation status. The 68 patients with adenocarcinoma having available data were stratified into four groups on the basis of tumor inflammation category (inflamed or noninflamed) and *TP53* mutation status (positive or negative). The longest RFS was apparent in patients with inflamed tumors and without *TP53* mutations (Fig. 3*D*). Collectively, these results suggested that the combination of tumor inflammation category and *TP53* mutation status might be associated with RFS in individuals with resected lung adenocarcinoma.

Discussion

Our results revealed that it is possible to identify patients with early-stage lung adenocarcinoma who are at increased risk of recurrence after adjuvant chemotherapy by taking into account PD-L1 expression, $CD8^+$ TIL density, and *TP53* mutation status. Immune checkpoint inhibitors are now being administered for postoperative therapy in lung cancer, prompting a reevaluation of the necessity for cytotoxic anticancer agents.

Although the classification of tumors on the basis of PD-L1 status and the presence of TILs has been proposed for other cancer types,[21,22] early-stage NSCLC has not previously been evaluated comprehensively for the relation between immune characteristics and clinical outcome. Many studies have analyzed PD-L1 expression as a potential prognostic factor in NSCLC, but the results have exhibited substantial variability.[8,23] We have now illustrated the potential use of including CD8 TIL^+ density together with PD-L1 TPS for the development of a

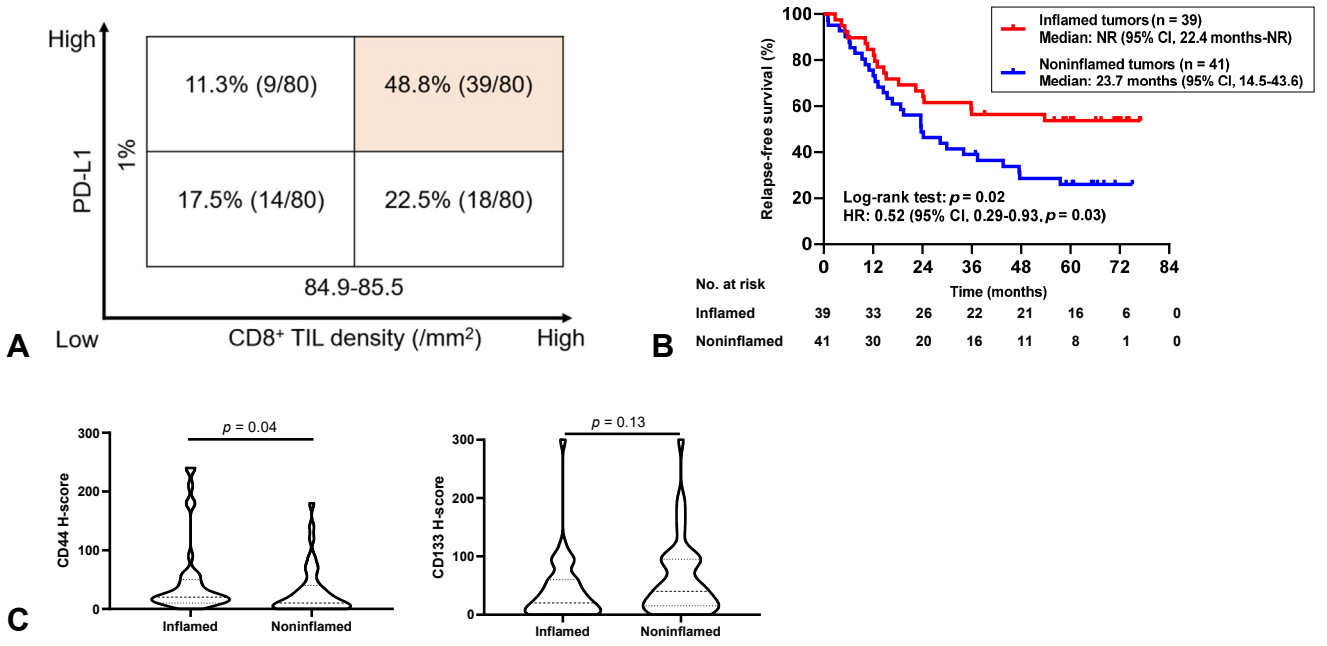

Figure 2. Classification of the tumor immune microenvironment on the basis of PD-L1 expression and $CD8^+$ tumor-infiltrating lymphocyte (TIL) density. (*A*) The tumor immune microenvironment for 80 patients with lung adenocarcinoma was classified according to cutoffs for the PD-L1 tumor proportion score and $CD8^+$ TIL density of 1% and 84.9-85.5/mm^2, respectively. Tumors with a PD-L1–positive and $CD8^+$ TIL density–high immune microenvironment were designated as inflamed, and all other tumors as noninflamed. (*B*) Kaplan-Meier curves for relapse-free survival of patients with inflamed tumors (n = 39) or noninflamed tumors (n = 41). (*C*) Violin plots of the CD44 and CD133 H-scores for inflamed (n = 39) and noninflamed (n = 41) tumors. Dashed and dotted lines indicate median and quartile values, respectively. The *p* values were determined with the Wilcoxon rank sum test. CI, confidence interval; NR, not reached; HR, hazard ratio; PD-L1, programmed death-ligand 1.

biomarker of recurrence. Furthermore, our findings suggest that the efficacy of immune checkpoint inhibitors might be limited in the patient subset at high risk for recurrence identified in our study.

The tumor suppressor protein TP53 plays multiple roles in the prevention or suppression of abnormal cell growth by inducing cell cycle arrest, apoptosis, or senescence and through control of cell metabolism and DNA repair.[24] Several studies have evaluated the relationship of *TP53* mutations to outcomes in individuals with NSCLC, but they have obtained conflicting results.[24–26] The tendency for the benefit of adjuvant chemotherapy to be greater for patients who are wild-type for *TP53* is consistent with the notion that the suppressor activities of the wild-type protein may contribute to an improved long-term benefit of such treatment. Conversely, the suggested association between *TP53* mutations and a worse outcome of adjuvant chemotherapy is consistent with a negative effect of some corresponding mutant proteins on the long-term therapeutic benefit.[26]

CSCs constitute a subpopulation of tumor cells that can drive tumor initiation and underlie tumor chemoresistance and recurrence.[11,27,28] CD44 is an adhesion molecule that interacts with hyaluronic acid and is implicated in a wide variety of physiological and pathologic processes.[29] High CD44 expression was found to be a negative prognostic marker for resected NSCLC.[30] CD133 is a transmembrane glycoprotein originally described in human hematopoietic stem cells, and its overexpression has been associated with poor survival in resected NSCLC.[16] Data on the relation of such CSC markers to chemotherapy outcome in early-stage NSCLC have been lacking, and the initial hypothesis of the present study was that CSC markers might be associated with the effectiveness of adjuvant chemotherapy as a result of the contribution of CSCs to therapeutic resistance. However, our data did not support this hypothesis. One complicating factor might be that, in individuals with early-stage NSCLC, unlike those with advanced-stage disease, chemotherapy is administered to prevent recurrence that might develop as a result of the presence of microscopic metastases. In addition, RFS tended to be shorter for patients with high versus low expression levels of CD44 or CD133, with the lack of statistical significance potentially attributable to the limited sample size.

PD-L1 expression has been associated with epithelial-mesenchymal transition, a transition to a CSC-like state, and PD-L1 has been found to induce this transition.[31] Expression of CD44 and CD133 might, therefore, be expected to exhibit a similar association with PD-L1 expression. CD44 was found to promote PD-L1

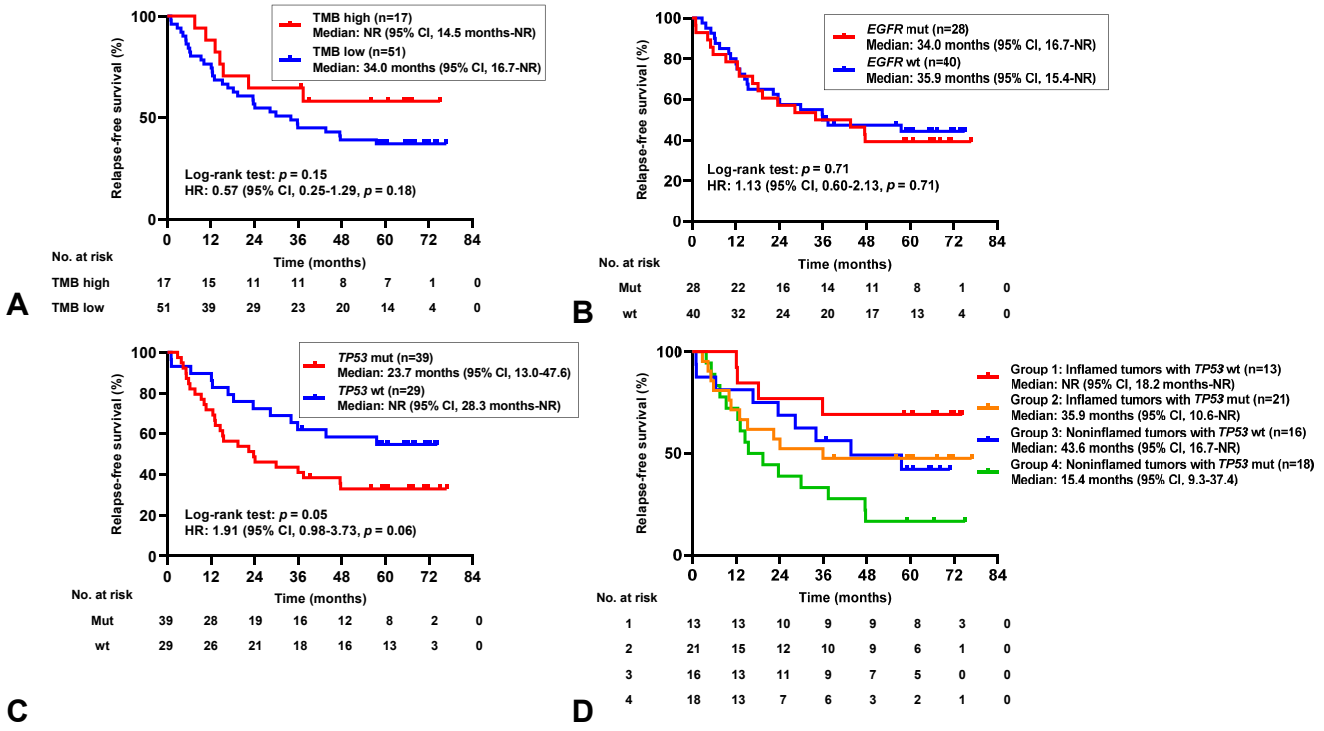

Figure 3. Kaplan-Meier curves for relapse-free survival of patients with lung adenocarcinoma classified on the basis of TMB (*A*), *EGFR* mutation status (*B*), *TP53* mutation status (*C*), or both tumor inflammation category and *TP53* mutation status (*D*). Patients were classified according to high or low TMB, mutant or wild-type status for *EGFR* or *TP53*, or inflamed versus noninflamed tumor category. CI, confidence interval; NR, not reached; HR, hazard ratio; TMB, tumor mutation burden; mut, mutant; wt, wild-type.

expression in NSCLC,[32] consistent with our present observation that CD44 expression was higher in tumors with a PD-L1 TPS of greater than or equal to 1%. As far as we are aware, the relation between CD133 and PD-L1 expression in NSCLC has not previously been evaluated. We found that CD133 expression tended to be higher in tumors with a PD-L1 TPS of less than 1%. We further evaluated the relation between CD44 or CD133 expression and PD-L1 expression according to tumor histologic type (Supplementary Fig. 5). In lung adenocarcinoma, CD44 and CD133 exhibited opposite associations with PD-L1 expression, whereas they manifested similar relations in other histologic types of the lung. These findings suggest that histologic differences may influence the association between CD44 or CD133 expression and PD-L1 expression.

Table 2. *EGFR* and *TP53* Mutation Status for Inflamed and Noninflamed Tumors

Mutation status	Inflamed (n = 34)	Noninflamed (n = 34)	*p* Value
EGFR mutation			0.08
Positive	10 (29.4)	18 (52.9)	
Negative	24 (70.6)	16 (47.1)	
TP53 mutation			
Positive	21 (61.8)	18 (52.9)	0.62
Negative	13 (38.2)	16 (47.1)	

Data are in number (percentage). The *p* values were determined with Fisher's exact test.

With regard to the relation between *EGFR* mutations and PD-L1 expression, EGFR signaling has been found to promote PD-L1 expression.[33] However, we found that PD-L1 expression tended to be lower in lung adenocarcinoma tumors with *EGFR* mutations than in those without such mutations (median TPS of 1 versus 20%, $p = 0.10$) (Supplementary Fig. 6*A*). The expression of CD44, which was found to promote PD-L1 expression in NSCLC,[32] was significantly higher in the tumors with *EGFR* mutations than in those without them (median H-score of 30 versus 10, $p = 0.001$) (Supplementary Fig. 6*B*). The observed trend for PD-L1 expression to be lower in *EGFR*-mutated tumors in our study might therefore be attributable to the small sample size (n = 68).

A strength of our study is that the RFS data were obtained prospectively for up to 5 years during the primary clinical trial (WJOG4107) and are, therefore, reliable. However, there are also limitations to the present study. First, it was retrospective in nature and the number of patients was relatively small, precluding multivariate analysis and analysis of a validation cohort. Second, the study population included only individuals who received adjuvant cytotoxic chemotherapy, with

there being no comparison cohort treated with adjuvant immunotherapy. Third, it remains unclear whether the cutoff value for $CD8^+$ TIL density (84.9–85.5/mm^2) is relevant. A previous study determined the optimal cutoff for $CD8^+$ TIL density (106/mm^2) by spatial analysis of TILs with an artificial intelligence model.[34] In the present study, TIL quantification was challenging as a result of the spatial bias of TILs across each slide and the heterogeneity of CD8 expression within individual tumors. Fourth, evaluation of the expression of each molecule was limited to quantitative assessment on the basis of IHC analysis, with the study, thus, lacking functional assessment and spatial analysis. It has recently become possible to assess functional PD-1–PD-L1 interaction (reflecting the interaction between TILs and tumor cells) and to analyze spatial information, with such data being potentially more useful than IHC findings as a biomarker.[35] Fifth, our study was conducted with an Asian population treated with S-1, and its findings cannot, therefore, be generalized to white populations.

In conclusion, our study indicates that cytotoxic anticancer agents may play a pivotal role in postoperative therapy for individuals with early-stage lung adenocarcinoma, especially for those with an inflamed TME. Further investigations are warranted to confirm whether the combination of tumor inflammation category and *TP53* mutation status might indeed serve as an effective biomarker of recurrence in such patients.

CRediT Authorship Contribution Statement

Hiroaki Kanemura: Conceptualization, Methodology, Investigation, Visualization, Writing-original draft, Writing-review and editing.

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Yasutaka Chiba: Investigation, Formal analysis, Writing-review and editing.

Tomoyuki Otani, Akihiko Ito, Kazuko Sakai, Kazuto Nishio: Methodology, Investigation, Writing-review and editing.

Nobuyuki Yamamoto, Isamu Okamoto, Kazuhiko Nakagawa: Supervision.

Masayuki Takeda: Conceptualization, Methodology, Investigation, Writing-review and editing, Supervision.

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Ethics Statement

All patients provided written informed consent, where applicable, or such informed consent was waived by institutional review board-approved protocols for aggregate deidentified data analysis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2024.100658.

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