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Lack of Association of Plasma Levels of Soluble Programmed Cell Death Protein 1, Programmed Death-Ligand 1, and CTLA-4 With Survival for Stage II to IIIA NSCLC After Complete Resection and Adjuvant Chemotherapy

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ABSTRACT

Introduction: Perioperative treatment in NSCLC has gained marked attention with the introduction of immune checkpoint inhibitors. Such a paradigm shift has given us additional opportunities to evaluate potential biomarkers in patients with these curable disease stages.

Methods: This study (WJOG12319LTR) was designed as a biomarker study to evaluate whether soluble immune markers were prognostic or predictive on relapse-free survival in patients with stage II to IIIA NSCLC who underwent complete resection and adjuvant chemotherapy with cisplatin plus S-1, which is an oral fluoropyrimidine formulation that consists of tegafur, gimeracil, and oteracil, or S-1 alone in the previous WJOG4107 study. Archived plasma samples were assayed for soluble (s) forms of programmed cell death protein 1 (sPD-1), programmed death-ligand 1(sPD-L1), and CTLA-4 (sCTLA-4) with the highly sensitive HISCL system. Using time-dependent receiver operating characteristic curve analysis, the area under the curves were derived and optimal cutoff values were determined. Using the cutoff values, whether the marker was prognostic or predictive was assessed by survival analysis.

Results: A total of 150 patients were included in the study. The time-dependent receiver operating characteristics analysis revealed that the area under the curves for sPD-1, sPD-L1, and sCTLA-4 were 0.54, 0.51, and 0.58, respectively. The survival analysis did not reject that hazard ratios were 1 in terms of the soluble immune marker and the treatment-marker interaction for all three markers.

Conclusions: There was no proof that circulating concentrations of sPD-1, sPD-L1, and sCTLA-4 were prognostic or predictive factors of the outcome for adjuvant chemotherapy after complete resection in patients with NSCLC.

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Introduction

Perioperative treatment in NSCLC has remained unchanged for decades; however, it has recently been receiving more attention than ever before. The reason for this is the reported improvement in prognosis with the introduction of immune checkpoint inhibitors (ICIs). Drugs that block immune checkpoint molecules such as programmed cell death 1 (PD-1), its ligand programmed death-ligand 1 (PD-L1), or CTLA-4 were initially developed for the treatment of cancer at advanced stages. The efficacy of these agents for tumors at earlier stages was subsequently exhibited, and they are becoming a component of the standard of care for early-stage cancer. In the case of NSCLC, the PD-L1 inhibitor atezolizumab was found to confer a survival benefit as adjuvant treatment after resection and platinum-based chemotherapy in patients with stage II to IIIA disease and a PD-L1 expression score of greater than or equal to 1% for tumor cells.[1] This finding led to the approval of atezolizumab by the U.S. Food and Drug Administration as the first immunotherapeutic agent for the treatment of NSCLC in the adjuvant setting. The PD-1 inhibitor pembrolizumab has also been approved for adjuvant treatment after resection and platinum-based chemotherapy for stage IB (T2a, ≥4 cm), II, or IIIA NSCLC.[2] These results have led to an increased focus on the exploration of biomarkers from diverse angles, including whether biomarkers identified from various perspectives, such as whether biomarkers discovered in the advanced stage of recurrence can be applied to the perioperative period, or whether biomarkers discovered in postoperative cytotoxic anticancer agents can be applied to perioperative immunotherapy.

Soluble forms of PD-1 (sPD-1) and PD-L1 (sPD-L1) have been detected in individuals with malignant or nonmalignant diseases. The soluble form of PD-1 is considered to be either produced through alternate splice variants or by shedding off the membrane form from cells that express PD-1, mainly activated T cells, and pro-B cells.[3] Elevated levels of sPD-1 and their association with disease activity have been exhibited for various inflammatory diseases.[4–7] The finding that sPD-1 interrupts the PD-1–PD-L1 interaction has suggested that this soluble factor might affect immunity.[7,8] sPD-L1 is also thought to be generated by proteolytic cleavage of the intact membrane-bound molecules or to be splice variants that are released from cells and enter the circulation.[7,9–12] It should be noted that a wide variety of cells, including nonmalignant cells such as dendric cells, can become a source of sPD-L1 as PD-L1 is expressed on various cells.[13] Levels of sPD-L1 were found to be increased and associated with poor outcomes in patients with cancer, including those with NSCLC.[14–17] Although

sCTLA-4 has also been detected and is thought to have an inhibitory effect on immune reactions,[18–20] less is known of its relation to disease than for sPD-1 and sPD-L1. sCTLA-4 could be produced by T cells, B cells, and macrophages as a form of splice variant.[18–20]

We have now performed a retrospective study (University hospital Medical Information Network database registration number UMIN000037723) to measure the circulating levels of sPD-1, sPD-L1, and sCTLA-4 and to evaluate their potential association with survival in patients with NSCLC who received adjuvant chemotherapy after complete tumor resection in a previously reported study (WJOG4107).[21]

Materials and Methods

Patients and Sample Collection

The present study (WJOG12319LTR) was undertaken as a biomarker study to evaluate soluble immune markers in stage II to IIIA NSCLC with the use of archived plasma samples from the WJOG4107 study (UMIN000001658). The design and results of WJOG4107, a randomized phase 2 study of adjuvant chemotherapy in stage II to IIIA NSCLC, have been described previously.[21] In brief, patients with completely resected stage II to IIIA NSCLC (classified according to the TNM staging system version 6) at an age of 20 to 74 years and with an Eastern Cooperative Oncology Group performance status of 0 or 1 and adequate bone marrow and organ function were eligible. The patients were randomly assigned to receive oral S-1 either alone or together with cisplatin. S-1 is an oral fluoropyrimidine formulation that consists of tegafur, gimeracil, and oteracil in a molar ratio of 1:0.4:1. The primary end points of WJOG4107 were relapse-free survival (RFS) at 2 years from the date of enrollment and identification of molecules whose expression was significantly associated with patient outcome. RFS events were defined as recurrence of disease or death for any reason and were 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 adjuvant therapy 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. Plasma samples were collected between the WJOG4107 enrollment completion and the initiation of postoperative adjuvant chemotherapy. The protocol specified initiation of treatment within 14 days of enrollment. Plasma samples were shipped to Kindai University Faculty of Medicine and archived under controlled temperature conditions of $-80°C$ until analysis. The present study
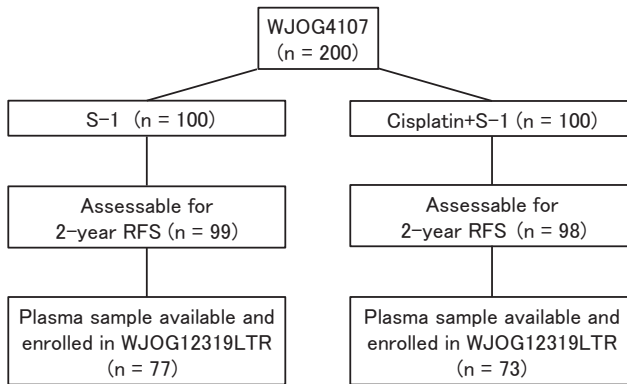
Figure 1. CONSORT diagram for WJOG12319LTR.

(WJOG12319LTR) was designed retrospectively after completion of the WJOG4107 trial and was approved by the ethics committee of each of the participating institutions. Among the patients enrolled in the WJOG4107 study, those for whom stored plasma samples were available were eligible for WJOG12319LTR (Fig. 1).

Measurement of Soluble Markers

Archival plasma samples were analyzed for sPD-1, sPD-L1, and sCTLA-4 with the use of a fully automated assay on the basis of chemiluminescence magnetic technology (HISCL system, Sysmex Corporation). The HISCL system is highly sensitive, reproducible, and precise for quantitative determination of the concentrations of these soluble immune markers in human plasma, as previously described.[22] The sample volume required for each assay was 20 mL.

Statistical Analysis

The primary interest of the current study was to assess whether soluble immune markers were prognostic or predictive of RFS. For the assessment, the time-dependent receiver operating characteristics (ROC) analysis at the 2-year time point was performed, in which area under the curve (AUC) was derived and the optimal cutoff point was determined for dividing patients into high and low groups of the biomarker. In each of the groups with high and low marker values, RFS curves for two treatment groups of S-1 monotherapy (S) and cisplatin plus S-1 combination therapy (SP) were drawn by the Kaplan-Meier method.[23] Whether the markers were prognostic or predictive was evaluated by the Cox proportional hazard regression model with three terms of treatment, marker, and these interactions as the explanatory variables, in which the marker term indicates whether the biomarker is prognostic and the interaction term indicates whether that is predictive.

For the other analyses, differences in continuous variables were assessed with the Mann-Whitney's U test and those in categorical variables with Fisher's exact test. Statistical analysis was performed with the use of Statistical Analysis System version 9.4 (by SAS Institute Inc., Cary, NC) and Easy R (developed in 2012 by Y. Kanda, Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (version 2.13.0, R Foundation for Statistical Computing, R Core Team, Vienna, Austria).

Results

Characteristics of the Study Population

Of the 200 patients treated in the WJOG4107 study, a total of 150 patients had plasma samples available, and were included in the WJOG12319LTR study (Fig. 1). The clinical characteristics of these patients are presented in Table 1. Among the 150 patients, 77 received S-1 monotherapy, whereas 73 received SP combination therapy. In this WJOG12319LTR study population, the 2-year RFS rate was 67.4% for the S-1 group versus 57.5% for the SP group, which was similar to the corresponding value for each treatment group of the WJOG4107 trial (65.6% version 58.1%, respectively).

Soluble Biomarkers and Clinical Characteristics

All patients had measurable baseline levels of sPD-1, sPD-L1, and sCTLA-4. The median (range) plasma concentrations of sPD-1, sPD-L1, and sCTLA-4 were 152.30 pg/mL (55.87–480.49 pg/mL), 177.62 pg/mL (105.60–426.78 pg/mL), and 1.33 pg/mL (0.63–6.39 pg/mL), respectively (Supplementary Fig. 1A–C). The relation of sPD-1, sPD-L1, and sCTLA-4 levels to clinicopathologic features is summarized in Table 2. Higher levels of sPD-L1 were observed in patients aged 65 years and older, in men, in patients with nonadenocarcinoma histological diagnosis, and current or former smokers compared with the corresponding paired subgroups. The levels of sCTLA-4 and sPD-1 did not reveal any apparent differences according to clinical characteristics.

Soluble Biomarkers and Survival

We first performed ROC curve analysis to evaluate the predictive value of the three soluble markers for RFS on the basis of the AUC. The AUC for sPD-1 to identify disease relapse at 2 years was 0.54. (Fig. 2A). The corresponding AUC values for sPD-L1 and sCTLA-4 were 0.51 and 0.58, respectively (Fig. 2B and C), suggestive of a poor discriminative ability for these parameters. The cutoff values obtained from the above time-dependent ROC analysis of sPD-1, sPD-L1, and sCTLA-4 were a value between 152.8 and 153.1 pg/mL, that

Table 1. Baseline Demographics and Disease Characteristics for the Study Patients

Characteristics	All, n (%)	S-1, n (%)	Cisplatin + S-1, n (%)
Median age (range), y	62 (37-74)	62 (37-74)	62 (38-74)
Sex			
Male	113 (75.3)	54 (70.1)	59 (80.8)
Female	37 (24.7)	23 (29.9)	14 (19.2)
Performance status			
0	94 (62.7)	55 (71.4)	39 (53.4)
1	41 (27.3)	18 (23.4)	23 (31.5)
Unknown	15 (10.0)	4 (5.2)	11 (15.0)
Histological diagnosis			
Adenocarcinoma	98 (65.3)	53 (68.8)	45 (61.6)
Nonadenocarcinoma	52 (34.7)	24 (31.2)	28 (38.4)
Pathologic stage			
II	83 (55.3)	43 (55.8)	40 (54.8)
IIIA	67 (44.7)	34 (44.2)	33 (45.2)
Type of surgery			
Lobectomy	150 (100)	77 (100)	73 (100)
Lymph node dissection			
ND0-1	7 (4.7)	4 (5.2)	3 (4.1)
ND2	143 (95.3)	73 (94.8)	70 (95.9)

ND, ND0-1 and ND2 designate no lymph node dissection, dissection to the N1 level or N2 level, respectively; S-1, oral fluoropyrimidine formulation that consists of tegafur, gimeracil, and oteracil.

between 183.8 and 184.1 pg/mL, and that between 1.42 and 1.43 pg/mL, respectively.

We next evaluated whether the soluble immune markers were prognostic or predictive of RFS in S-1 monotherapy version SP therapy using the above cutoff values. Kaplan-Meier plots of S and SP are illustrated in Figure 3, in each of the groups with the low and high sPD-1 (Fig. 3A and B), sPD-L1 (Fig. 3C and 3D), and

sCTLA-4 (Fig. 3E and F). For sPD-1, the hazard ratio for the marker term (high/low) was 1.19 (95% confidence interval [CI], 0.62–2.28; $p = 0.59$), and that for the treatment-marker interaction term was 1.39 (95% CI: 0.56–3.47; $p = 0.48$). The corresponding hazard ratios for sPD-L1 were 0.75 (95% CI: 0.39–1.43; $p = 0.37$) and 1.01 (95% CI: 0.38–2.63; $p = 0.99$), and those for sCTLA-4 were 1.56 (95% CI: 0.82–2.90; $p = 0.18$) and

Table 2. Plasma Levels of Soluble Markers (pg/mL) According to Patient Characteristics

Characteristics	n (%)	sPD-1 median (range)	p	sPD-L1 median (range)	p	sCTLA-4 median (range)	p
Age (y)							
<65	87 (58.0)	149.96 (55.87-480.49)	0.317	162.48 (112.43-426.78)	**0.005**	1.34 (0.63-6.39)	0.708
≥65	63 (42.0)	158.01 (95.46-380.80)		185.03 (105.60-382.30)		1.32 (0.83-1.62)	
Sex							
Male	113 (75.3)	150.60 (55.86-480.49)	0.619	181.22 (159.17-382.30)	**0.002**	1.32 (0.63-3.68)	0.737
Female	37 (24.6)	156.52 (70.43-380.83)		157.96 (105.60-426.78)		1.36 (0.84-6.39)	
Smoking status							
Current/former	113 (75.3)	150.67 (55.87-480.49)	0.676	178.78 (116.94-382.30)	**0.038**	1.34 (0.84-6.39)	0.858
Never	37 (24.6)	156.52 (95.46-380.8)		159.24 (105.60-426.78)		1.32 (0.84-2.99)	
Performance status							
0	94 (62.7)	150.73 (126.77-188.35)	0.610	174.54 (152.47-194.61)	0.802	1.37 (1.14-1.58)	0.454
1	41 (27.3)	156.98 (119.84-210.91)		177.23 (154.78-197.10)		1.32 (1.15-1.55)	
Histological diagnosis							
Adenocarcinoma	98 (65.3)	154.42 (80.87-480.49)	0.471	173.14 (105.60-426.78)	**0.007**	1.34 (0.84-6.39)	0.588
Nonadenocarcinoma	52 (34.7)	150.35 (55.86-285.84)		190.32 (125.93-373.91)		1.33 (0.63-2.47)	
Pathologic stage							
II	83 (55.3)	153.45 (55.87-480.49)	0.664	178.56 (112.44-382.30)	0.744	1.31 (0.63-6.39)	0.487
IIIA	67 (44.7)	150.79 (80.87-314.27)		173.95 (105.60-426.78)		1.36 (0.85-2.14)	

Note: The p values were determined with Fisher's exact test, and those of less than 0.05 are illustrated in bold.
sCTLA-4, soluble CTLA-4; sPD-1, soluble programmed cell death protein 1; sPD-L1, soluble programmed death-ligand 1.
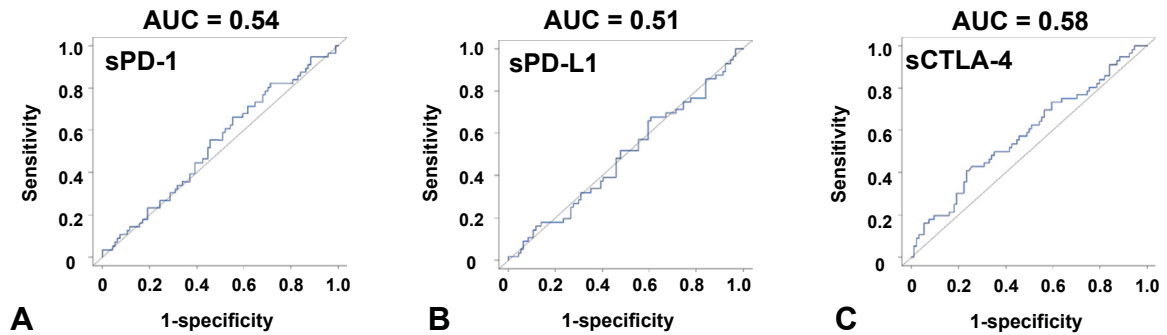
Figure 2. ROC curves for prediction of 2-year RFS on the basis of the plasma levels of sPD-1 (*A*), sPD-L1 (*B*), or sCTLA-4 (*C*). AUC, area under the ROC curve; RFS, relapse-free survival; ROC, receiver operating characteristic; sCTLA-4, soluble CTLA-4; sPD-1, soluble programmed cell death protein 1; sPD-L1, soluble programmed death-ligand 1.

0.86 (95% CI: 0.34–2.20; $p = 0.75$). The results did not suggest that these three soluble markers were prognostic or predictive of RFS in S monotherapy versus SP.

We finally assessed the diagnostic accuracy of the three markers for RFS at different time points up to 5 years (Fig. 4A–C). None of the markers exhibited an AUC of greater than 0.6 at any time point between 2 and 5 years. The results did not suggest that plasma levels of sPD-1, sPD-L1, and sCTLA-4 were associated with RFS in the population evaluated in this study.

Discussion

Adjuvant therapy after curative surgery for NSCLC had remained unchanged for many years until the recent introduction of ICIs for such early-stage disease.[1,2] In addition to postoperative therapy, preoperative ICI treatment has been found to be effective,[24,25] with optimal patient selection for clinical use still to be determined. For NSCLC at advanced stages, clinical biomarkers for ICI treatment include PD-L1 expression on tumor cells, tumor mutation burden (TMB), and microsatellite instability status. The clinical benefit of adjuvant atezolizumab for stage II to IIIA NSCLC was most

Figure 3. Kaplan-Meier curves for RFS as determined by the levels of sPD-1 (*A, B*), sPD-L1 (*C, D*), and sCTLA-4 (*E, F*) classified according to adjuvant chemotherapy group. Vertical bars denote censoring, and *p* values were determined with the log-rank test. CI, confidence interval; HR, hazard ratio; NR, not reached; RFS, relapse-free survival; sCTLA-4, soluble CTLA-4; sPD-1, soluble programmed cell death protein 1; sPD-L1, soluble programmed death-ligand 1.

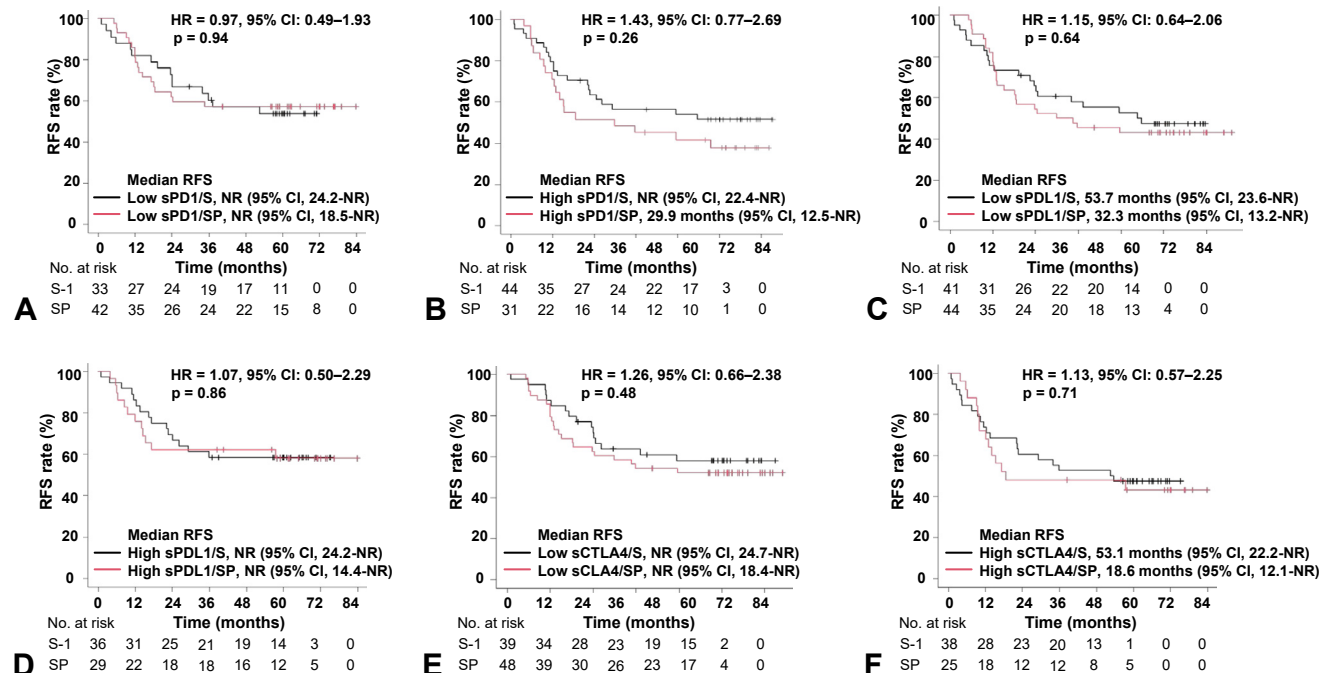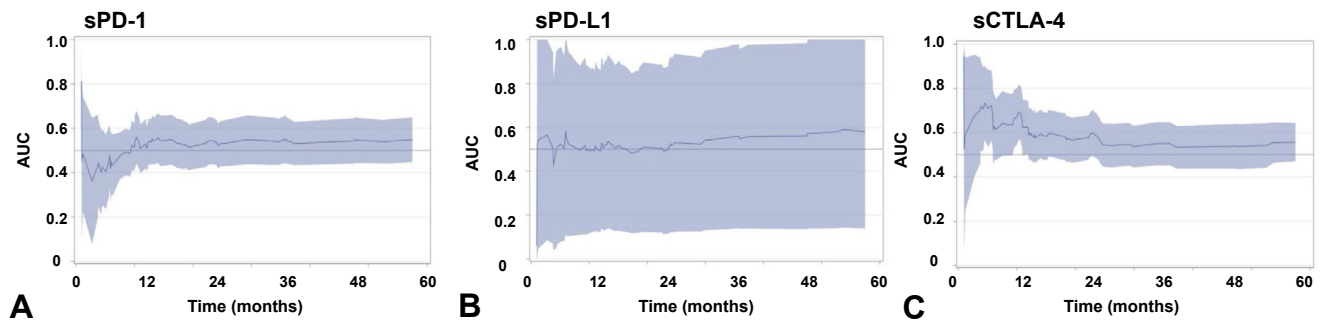**Figure 4.** Time-dependent ROC analysis for RFS on the basis of the plasma levels of sPD-1 (*A*), sPD-L1 (*B*), or sCTLA-4 (*C*) with 95% CI (colored areas). AUC, area under the ROC curve; CI, confidence interval; RFS, relapse-free survival; ROC, receiver operating characteristic; sCTLA-4, soluble CTLA-4; sPD-1, soluble programmed cell death protein 1; sPD-L1, soluble programmed death-ligand 1.

pronounced for patients with a PD-L1 expression score of 50% or greater,[1] suggesting the potential usefulness of this marker for prediction of ICI response regardless of disease stage. It should be noted that PD-L1 expression has been found to be neither a prognostic nor a predictive factor for adjuvant cytotoxic chemotherapy in NSCLC,[26] suggesting that its use depends on the specific treatment. TMB has been identified as a prognostic factor in resectable NSCLC, with the benefit of adjuvant chemotherapy being greater for tumors with a low TMB, which have a poorer prognosis.[27] Thus, some factors are biomarkers of survival after complete resection irrespective of the type of drug treatment, whereas others are not. In the search for biomarkers for perioperative therapy, it is, therefore, important to evaluate the association between biomarker candidates and the various agents that are used in perioperative treatment options.

Exploration of liquid biomarkers has been performed in addition to that of tissue-based biomarkers. Various cell membrane–bound proteins have been found to exist also in soluble forms, most of which are generated by proteolytic cleavage. The association of a high sPD-L1 level with poor prognosis, including a shorter overall survival or shorter progression-free survival during ICI treatment, has been exhibited in several cancer types.[28–31] Circulating levels of PD-1 and PD-L1 do not correlate with tissue expression levels of the corresponding membrane-bound proteins,[28,29,32] making these factors to be considered as independent biomarkers from those assessed by tissue samples. In addition, dynamic changes in sPD-L1 concentration after ICI treatment have also been described and may reflect the immune status of the patient at each time point.[29] We recently found that high concentrations of sPD-1, sPD-L1, and sCTLA-4 were associated with overactivation or hyperexhaustion of antitumor immunity in patients with NSCLC, and that combination of the levels of these soluble factors with that of PD-L1 expression in tumor tissue allowed more precise patient selection with regard to the predicted response to

ICI treatment (article under revision). A recent study has found that a soluble form of PD-L1 can act as a decoy of anti–PD-L1 antibody treatment, leading to resistance to such therapy.[11] Such increasing findings obtained in the advanced stage led us to explore the role of these factors as biomarkers in the resectable stage. In the present study, we found that sPD-1, sPD-L1, and sCTLA-4 levels were not prognostic or predictive factors of the outcome for postoperative adjuvant chemotherapy in patients with stage II to IIIA NSCLC. These results provide important background information for future investigations of the role of such soluble factors in postoperative therapies, including those with immunotherapy.

Our study has some limitations. First, the study population included only individuals who received adjuvant cytotoxic chemotherapy; there was no comparison cohort treated with adjuvant ICI therapy. Second, the relation of the soluble immune markers to the tumor immune environment was not assessed. Third, there are no established cutoff levels of sPD-1, sPD-L1, and sCTLA-4, even for cancer at advanced stages. However, 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, unlike those of purely retrospective studies.

In conclusion, our results indicate that circulating levels of sPD-1, sPD-L1, and sCTLA-4 are not predictive or prognostic of outcome for adjuvant chemotherapy in patients with completely resected NSCLC. Further studies are warranted to explore whether these soluble factors are potential biomarkers in patients receiving postoperative ICI therapy.

CRediT Authorship Contribution Statement

Junko Tanizaki: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft, Writing-review and editing.

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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.2023.100590.

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