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Original Article

Title

High expression of programmed cell death 1 ligand 1 in lung adenocarcinoma is a poor prognostic factor particularly in smokers and wild-type epidermal growth-factor receptor cases

Running title PD-L1 expression in lung adenocarcinoma

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Abstract

A clinical implication of programmed cell death 1 ligand 1 (PD-L1) expression in lung adenocarcinoma has not been well established. We evaluated PD-L1 expression immunohistochemically on 296 surgically resected lung adenocarcinomas to investigate a clinical implication of PD-L1 expression especially in terms of smoking history and epidermal growth-factor receptor (EGFR) mutation status. Patients were classified into high- and low-PD-L1 expression groups. The high-expression group (n = 107) showed a significantly higher proportion of smokers and poor differentiation compared with the low-expression group (n = 189). Survival analysis showed that the prognosis of the high-expression group was worse in overall survival than that of the low-expression group (3-year overall survival 85 vs. 94 %, p = 0.005). Stratified survival analyses showed that the prognoses of the high-expression group were worse than those of the low-expression group in both strata of smokers and wild-type EGFR (p = 0.009 and p = 0.007, respectively). We found that high PD-L1 expression was a poor prognostic factor in the smokers or the patients with wild-type EGFR, whereas it was not the case in those who never smoked or those with EGFR mutation, implying the importance of adenocarcinoma driver mutations and etiology.

Keywords

adenocarcinoma, EGFR, Immunohistochemistry, lung cancer, programmed cell death 1 ligand 1, smoking

Text

INTRODUCTION

Immune-checkpoint therapy targeting programmed cell death 1 (PD-1) and one of its ligands, programmed cell death 1 ligand 1 (PD-L1), is a new therapeutic strategy for patients with cancer. PD-L1 binding to PD-1 expressing on the surface of T-cells suppresses activation and proliferation of T-cells. Many types of cancer frequently overexpress PD-L1 and escape the host immune system, and monoclonal antibodies for PD-1 or PD-L1 have been expected as one of the breakthrough immune check-point therapies.[1, 2] Among non-small cell lung cancer (NSCLC), favorable outcomes of therapies using these antibodies have been reported even in tumors of advanced stages.[3-6]

Relationships between PD-L1 expression on tumor cells and therapeutic response have been suggested among NSCLC.[6, 7] Some of the studies have reported that PD-L1 expression is a prognostic factor and relevant to gender, smoking history, epidermal growth-factor receptor (EGFR) status, and tumor differentiation.[8-11] However, the utility as a predictive biomarker and a clinical implication of PD-L1 expression have not yet been well established.

Many studies have analyzed the NSCLC as a single category, there being only a few studies specifically analyzing lung adenocarcinoma. We presume that because adenocarcinoma and squamous cell carcinoma have very different characteristics in terms of etiology and driver mutation status, the two types of tumors should be analyzed differently. Therefore, we herein evaluated the PD-L1

expression and investigated a clinical implication by focusing on the adenocarcinoma histology in terms of smoking history and EGFR mutation status.

MATERIALS AND METHODS

Patient selection and data collection

Patients who underwent surgical resection for lung adenocarcinoma between May 2009 and November 2013 at the Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan, were consecutively selected. Those who received neoadjuvant chemotherapy and/or radiotherapy, underwent limited resection such as segmentectomy or wedge resection, and were diagnosed with adenocarcinoma in situ or minimally invasive adenocarcinoma were excluded. Histological diagnosis was made by three authors (S.M, N.M. and Y.I.) including lung expert pathologists, based on the 2015 World Health Organization classification[12] and differentiation grades were determined according to our previous study.[13] Histological subtypes and differentiation grades were determined, based on whole sections of each tumor, not on tissue arrays. Therefore, we enrolled 296 patients with overt invasive adenocarcinoma treated by complete resection for analysis of this study.

Clinicopathological factors including age, sex, smoking history, histological subtypes, differentiation grades, EGFR mutation status, pathological T and N factors, lymphatic invasion, vascular invasion, platinum-based adjuvant

chemotherapy, treatment with EGFR-TKI (tyrosine kinase inhibitor) and follow-up information were collected from medical records. For the cases, mutation data of which were not available in the medical record, we here newly examined EGFR exon 19 deletion by the fragment analysis using the ABI PRISM fluorescence primer method and L858R mutation by the genotyping assay using Custom TaqMan SNP Genotyping Assays. We confirmed causes of death using death certificates in deceased cases. Disease-free survival (DFS) was defined as the time between the date of the surgery and local recurrence, distant metastasis, or death, which ever occurred first. Overall survival (OS) was defined as the time between the date of the surgery and death.

All procedures performed in this study were in accordance with the ethical standards of the institutional review board and with the Declaration of Helsinki and its later amendments. Informed consent was obtained from all patients included in the study by comprehensive informed consent forms.

Evaluation of PD-L1 expression

We used tissue microarrays (TMA) from formalin-fixed paraffin-embedded surgical specimens of all 296 patients for evaluation of PD-L1 immunohistochemical expression. Samples for TMA were collected from a spot of tumors with the most representative histology of each surgical specimen with 2-mm diameter core needles. The representative histology is the same as the predominant component for some cases and it contains the most predominant and the second predominant components for other cases. The samples were

arrayed in a new paraffin block. TMA sections at 4-μm thickness mounted on silane-coated slides were routinely deparaffinized in xylene and dehydrated in a graded ethanol series. For antigen retrieval, the slides were heated at 97˚C for 40 minutes in citrate buffer at pH 6.0 or for 20 minutes in ethylenediaminetetraacetic acid buffer at pH 9.0. The slides were stained for PD-L1 with anti-CD274 (PD-L1) antibody, rabbit monoclonal (clone EPR1611, Abcam plc, Cambridge, UK) on an automated staining platform (BOND III; Leica Biosystems Melbourne Pty Ltd, Australia) using a concentration of 1:200. Immunohistochemical staining was performed between December 2014 and June 2015.

Without linking to patients' information, we evaluated stain intensity (0, negative; 1, weakly positive; 2, strongly positive) and percentages of tumor cells with each stain intensity. We defined the sum of products from multiplying the percentages by the stain intensity (0, 1, or 2) as a PD-L1 score. For example, in case of weakly positive results of 30 % and strongly positive of 20 %, the PD-L1 score was 1x30+2x20= 70. We sought a reasonable cutoff value of PD-L1 expression to classified patients into high- and low-PD-L1 expression group. We compared difference of OS between two groups divided by various exploratory cutoff values, with regardless intensity or with PD-L1 score considering intensity, and adopted the cutoff value showed most significant difference.

Statistical analyses

We compared clinicopathological characteristics between the high- and low-PD-L1 expression groups. Fisher's exact test or chi-square test was used to

assess the correlations between variables and between the groups, as appropriate. Multivariate logistic regression analysis was used to determine independent predictive factors of high PD-L1 expression among patients' clinicopathological variables.

The prognostic differences between the high and low groups were analyzed. Analyses stratified by smoking history and the EGFR status were also performed. Survival curves were generated using the Kaplan–Meier method. DFS and OS were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model to identify independent prognostic factors for OS. We used IBM® SPSS® Statistics version 19 (Chicago, IL, USA) to perform the statistical analyses. Significance was defined as $p < 0.05$.

RESULTS

Clinicopathological characteristics of the high- and low-PD-L1 expression groups

In total, 296 patients with overt invasive adenocarcinoma were included in this study. First, we determined a cutoff value to classify the cases into high- and low-expression groups. Exploratory cutoff values were set as 10, 20, 50 % of positive tumor cells without consideration of intensity as well as 10, 20, 50, 80 PD-L1 score with consideration of intensity, and then differences of OS between two groups were calculated and *p* values were compared. As a result, *p* values

were 0.263, 0.014, 0.015 and 0.263, 0.016, 0.005, 0.012, respectively, showing the lowest *p* value (*p*=0.005) when using the cutoff value of 50 PD-L1 score, considering intensity. Hence, we adopted the 50 PD-L1 score as a cutoff value. For all cases, the median and standard deviation (SD) of the PD-L1 score were 20 and 47, respectively. By the score, 107 patients (36 %) were classified into the high-expression group with median = 90 and SD = 36, and the remaining 189 patients (64 %) were classified as the low-expression group with median = 10 and SD = 13. Fig. 1 gives representative pictures for high- and low-PD-L1 expression cases.

Table 1 shows a comparison of patients' clinicopathological characteristics between the high- and low-PD-L1 expression groups. In the high-PD-L1 expression group (n=107), 28 patients (26%) developed recurrence, and 11 of them had EGFR mutation and 9 patients were treated with EGFR-TKI. In the low-PD-L1 expression group (n=189), tumors of 36 patients (19%) recurred, and 20 of them were EGFR-mutated and 18 were treated with EGFR-TKI. The high-expression group showed a significantly higher proportion of current/former smokers and poorly differentiated adenocarcinoma compared with the low-expression group (p = 0.008 and p = 0.018, respectively). There were no significant differences in age, gender, histological subtypes, EGFR status, pathological T and N factors, lymphatic invasion, vascular invasion, platinum-based adjuvant chemotherapy, and treatment with EGFR-TKI.

As shown in Table 2, multivariate logistic regression analysis for predictors of high PD-L1 expression revealed that only the current/former smoking was an

independent predictive factor for high PD-L1 expression (odds ratio = 1.884; 95 % confidence interval 1.117–3.180; *p* = 0.018).

Survival analyses of the high- and low-PD-L1 adenocarcinomas

The median and SD of follow-up period was 31 and 12 months, respectively. Fig. 2 shows a comparison of the Kaplan–Meier curves of OS and DFS of the high- and low-PD-L1 expression groups. In both OS and DFS, the prognosis of the high-expression group was significantly worse than that of low-expression group. The 3-year OS for the high- and low-expression group was 85 vs. 94 % (*p* = 0.005), and the DFS was 69 vs. 81 % (*p* = 0.021), and both differences were significant. The univariate analysis of OS shown in Table 3 indicates that the acinar/micropapillary/solid histological subtype, moderately/poorly differentiation, pT2-4, and pN1-2 were worse prognostic factors in addition to the high PD-L1 expression. The multivariate Cox regression analysis for OS shown in Table 4 indicates that the high PD-L1 expression, as well as the pN1-2 and the moderately/poorly differentiation, was an independent prognostic factor (hazard ratio = 2.668; 95% confidence interval 1.275–5.581; *p* = 0.009).

Fig. 3 shows the Kaplan–Meier curves of OS stratified by smoking (Fig. 3a) and EGFR mutation status (Fig. 3b). Upon stratified analyses, Supplementary Table 1 shows patients' smoking history and EGFR status with cross tabulation. In order to estimate the impact of EGFR-TKI on the 64 cases with recurrence, OS was compared between the 27 patients treated with EGFR-TKI therapy and the 37 patients without the therapy. Although 3-year OS was 77 % and 58%

respectively, there was no statistical significance (p = 0.800). In the stratum of current/former smokers (n = 154), the high-PD-L1-expression group (n = 67) showed significantly worse prognosis than the low-expression group did (n = 87), their 3-year OS being 80 vs. 94 % (p = 0.009). In contrast, there was no difference in the stratum of those who never smoked (n = 142) between the two groups, their 3-year OS being 94 vs. 94 % (p = 0.489), which is not significant. In the stratum of the wild-type EGFR (n = 160), the high-expression group (n = 61) showed significantly worse prognosis compared with the low-expression group (n = 99), their 3-year OS being 81 vs. 94 % (p = 0.007). On the contrary, there was no difference in the stratum of mutant EGFR (n = 136) between the two groups, their 3-year OS being 90 vs. 94 % (p = 0.423).

DISCUSSION

By focusing on adenocarcinoma histology, rather than non-small cell carcinoma, we successfully revealed that high PD-L1 expression was a prognostic factor, and the prognostic significances were particularly remarkable in smokers and EGFR wild type tumor.

Some studies pointed out a relation between smoking and PD-L1 expression in NSCLC. Calles et al. suggested that PD-L1 expression was induced by smoking in KRAS mutant NSCLC.[10] Deng et al. reported that those who had smoked had elevated gene expression of PD-L1 compared with those who never smoked.[14] These results were consistent with ours. On the contrary, Azuma et al.

reported that, although PD-L1 expression was higher in those who never smoked than in smokers according to their univariate analysis, the multivariate analysis demonstrated that smoking status was not an independent predictor for high PD-L1 expression.[9] We think that this discrepancy is probably due to their analysis by admixing adenocarcinoma and squamous cell carcinoma, considering almost all squamous cell carcinomas arise in smokers.

Although our study did not show any significant difference of the EGFR mutation rate between the high- and low-PD-L1 groups, D'Incecco et al. reported that PD-L1 immunoreactivity was associated with adenocarcinoma histology and the presence of EGFR mutations in NSCLC in their univariate analysis.[8] Again, we suppose this correlation may be caused by their admixture of adenocarcinoma with frequent EGFR mutations and squamous cell carcinoma with almost no such mutations. Nevertheless, we note that there was a report, showing by the multivariate analysis, that the mutant status of EGFR was an independent predictive factor for high PD-L1 expression,[9] which is not in line with our results.

There are many studies with various modalities, reporting that the high PD-L1 expression was a poor prognostic factor in NSCLC. Azuma et al. employed immunohistochemistry,[9] Deng et al. used microarray datasets,[14] and Ikeda et al. investigated copy number alterations of the PD-L1 gene using real-time PCR.[15] All of those studies reported that the high PD-L1 expression was a poor prognostic factor. Pan et al., in their meta-analysis, also indicated that the PD-L1 expression was a factor of unfavorable prognosis for NSCLC.[11] These findings are consistent with our results, and, additionally, our study revealed that

adenocarcinomas with high PD-L1 expression had a less favorable outcome for both OS and DFS. Moreover, and notably, we successfully demonstrated that such poorer prognosis was particularly remarkable in smokers and cases without EGFR mutations.

This study has some limitations. First, we evaluated only PD-L1 protein expression. Obviously, we should examine various kinds of immune cells,[10, 16] such as CD4 (helper), CD8 (cytotoxic), CD25 (regulatory), CD68 (macrophages), and other PD-L family proteins, to understand actual roles of tumor immunity. Second, we employed TMA rather than large sections. There was the fact that TMA contained only the predominant component for some cases or two components (the most predominant and the second predominant components) for others. Because there certainly is heterogeneity of PD-L1 expression in most of tumors, a study on how different the PD-L1 expression between TMA and whole sections would be warranted, bearing in mind a report on underestimation of PD-L1 expression by biopsy materials as compared with whole tissues.[17] Third, PD-L1 evaluation by immunohistochemistry have not been standardized yet. We do not know if the antibody used in this study is appropriate in terms of specificity and sensitivity, and there is no established definition of positivity. These are currently unresolved issues.[18, 19]

In conclusion, we revealed that the high PD-L1 expression was a poor prognostic factor in smokers or cases with wild-type EGFR, whereas it was not the case in the subgroups of those who never smoked or those with EGFR mutation in lung adenocarcinoma. The findings suggest that the clinical implication of PD-L1 expression is different in each group classified by etiology. In

immune-checkpoint therapy, PD-L1 expression as a predictor of therapeutic response is still controversial, and, therefore, a more effective predictor is required. Our findings suggest that when analyzing the relation between PD-L1 expression and outcome of immune-checkpoint therapy, the oncologic background of lung adenocarcinoma patients, such as driver mutation and smoking history, should be considered.

ACKNOWLEDGMENTS

We thank Hiroko Nagano and Eru Adachi for making the tissue microarrays, Tomoyo Kakita for immunohistochemical staining, Motoyoshi Iwakoshi for slicing tissue blocks, Miyuki Kogure for preparation of slides, and Kentaro Inamura for critical reading of the manuscript. Parts of this study were supported financially by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from the Japan Society for the Promotion of Science; the Ministry of Health, Labour and Welfare; and the Smoking Research Foundation.

DISCLOSURE STATEMENT

None declared.

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Table 1 Clinicopathological characteristics of patients with lung adenocarcinoma classified by PD-L1 expression

	PD-L1 high-expression group		PD-L1 low-expression group		*p* value
	n = 107	(%)	*n* = 189	(%)	
Age					0.535
<70	63	(59)	119	(63)	
≥70	44	(41)	70	(37)	
Gender					0.090
Male	61	(58)	87	(46)	
Female	46	(43)	102	(54)	
Smoking history					0.008*
Current/former	67	(63)	87	(46)	
Never	40	(37)	102	(54)	
Histological subtypes					0.058
Lepidic	13	(12)	23	(12)	
Papillary	67	(62)	140	(75)	
Acinar	5	(5)	10	(5)	
Micropapillary	3	(3)	2	(1)	
Solid	19	(18)	14	(7)	
Differentiation					0.018*
Well	36	(34)	76	(40)	
Moderately	49	(46)	96	(51)	
Poorly	22	(20)	17	(9)	
EGFR status					0.468
Mutant	46	(43)	90	(48)	
Wild-type	61	(57)	99	(52)	
T factor					0.730
T1	51	(48)	100	(53)	
T2	42	(39)	71	(38)	
T3	12	(11)	16	(8)	
T4	2	(2)	2	(1)	
N factor					0.756
N0	78	(73)	145	(76)	
N1	15	(14)	22	(12)	
N2	14	(13)	22	(12)	
Lymphatic invasion					1.000
Yes	51	(48)	89	(47)	

No	56	(52)	100	(53)	
Vascular invasion					0.396
Yes	61	(57)	97	(51)	
No	46	(43)	92	(49)	
Platinum-based adjuvant chemotherapy					1.000
Yes	9	(8)	15	(8)	
No	98	(92)	174	(92)	
Recurrence					0.186
Yes	28	(26)	36	(19)	
No	79	(74)	153	(81)	
Treatment with EGFR-TKI					0.836
Yes	9	(8)	18	(10)	
No	98	(92)	171	(90)	

*Significance at $p < 0.05$

Table 2 Multivariate logistic regression analysis for predictors of high PD-L1 expression

Factor	Variable	Odds ratio	95% confidence interval	*p* value
Smoking	Never	Ref		
	Current/former	1.884	1.117 - 3.180	0.018*
Histological subtypes	Papillary	Ref		
	Lepidic	1.286	0.289 - 6.948	0.768
	Acinar	0.822	0.366 - 1.849	0.636
	Micropapillary	0.707	0.175 - 2.853	0.626
	Solid	2.796	0.386 - 20.258	0.309
Differentiation	Well	Ref		
	Moderately	1.021	0.566 - 1.842	0.945
	Poorly	1.697	0.390 - 7.386	0.481
EGFR status	Wild-type	Ref		
	Mutated	1.090	0.644 - 1.844	0.747

*Significance at $p < 0.05$

Table 3 Univariate analysis of overall survival of 296 patients with overt invasive adenocarcinoma

Variable	*n*	(%)	3-year OS (%)	*p* value
PD-L1 expression				0.005*
Low	189	(64)	94	
High	107	(36)	85	
Age				0.067
<70	182	(61)	93	
≥70	114	(39)	89	
Gender				0.673
Male	148	(50)	91	
Female	148	(50)	91	
Smoking history				0.235
Current or former	154	(52)	88	
Never	142	(48)	94	
Histological subtypes				0.003*
Lepidic/papillary	242	(82)	95	
Acinar/micropapillary/solid	54	(18)	72	
Differentiation grades				< 0.001*
Well	112	(38)	99	
Moderately/poorly	184	(62)	86	
EGFR status				0.546
Mutant	136	(46)	93	
Wild-type	160	(54)	89	
T factor				0.001*
T1	151	(51)	96	
T2-4	145	(49)	85	
N factor				< 0.001*
N0	223	(75)	95	
N1-2	73	(25)	79	

*Significance at $p < 0.05$

Table 4 Multivariate Cox regression analysis of overall survival of 296 patients with invasive adenocarcinoma

Variable	Hazard ratio	95% confidence interval			*p* value
Moderately/poorly differentiation	5.430	1.167	-	25.274	0.031*
Acinar/micropapillary/solid predominant	1.071	0.468		2.449	0.871
pT2-4	2.323	0.919	-	5.869	0.075
pN1-2	2.424	1.067	-	5.509	0.035*
High PD-L1 expression	2.594	1.248	-	5.393	0.011*

*Significance at $p < 0.05$

FIGURE LEGENDS

Figure 1 Representative pictures of high- (a, b) and low- (c) PD-L1 expression groups evaluated with the PD-L1 score by immunohistochemical staining: (a) papillary adenocarcinoma presenting a score of 120; (b) solid adenocarcinoma presenting a score of 150; (c) papillary adenocarcinoma presenting a score of 5.

Figure 2 Kaplan–Meier curves of overall survival (OS) and disease-free survival (DFS) as compared between the high- (*n* = 107) and low- (*n* = 189) PD-L1 expression groups. Note that the high-expression group has significantly unfavorable prognosis for both OS and DFS.

Figure 3 Kaplan–Meier curves of overall survival stratified by smoking history (a) and EGFR status (b) between the high- and low-PD-L1-expression groups.

List of supplementary material

Supplementary Table 1 Smoking history and EGFR status

Figure1

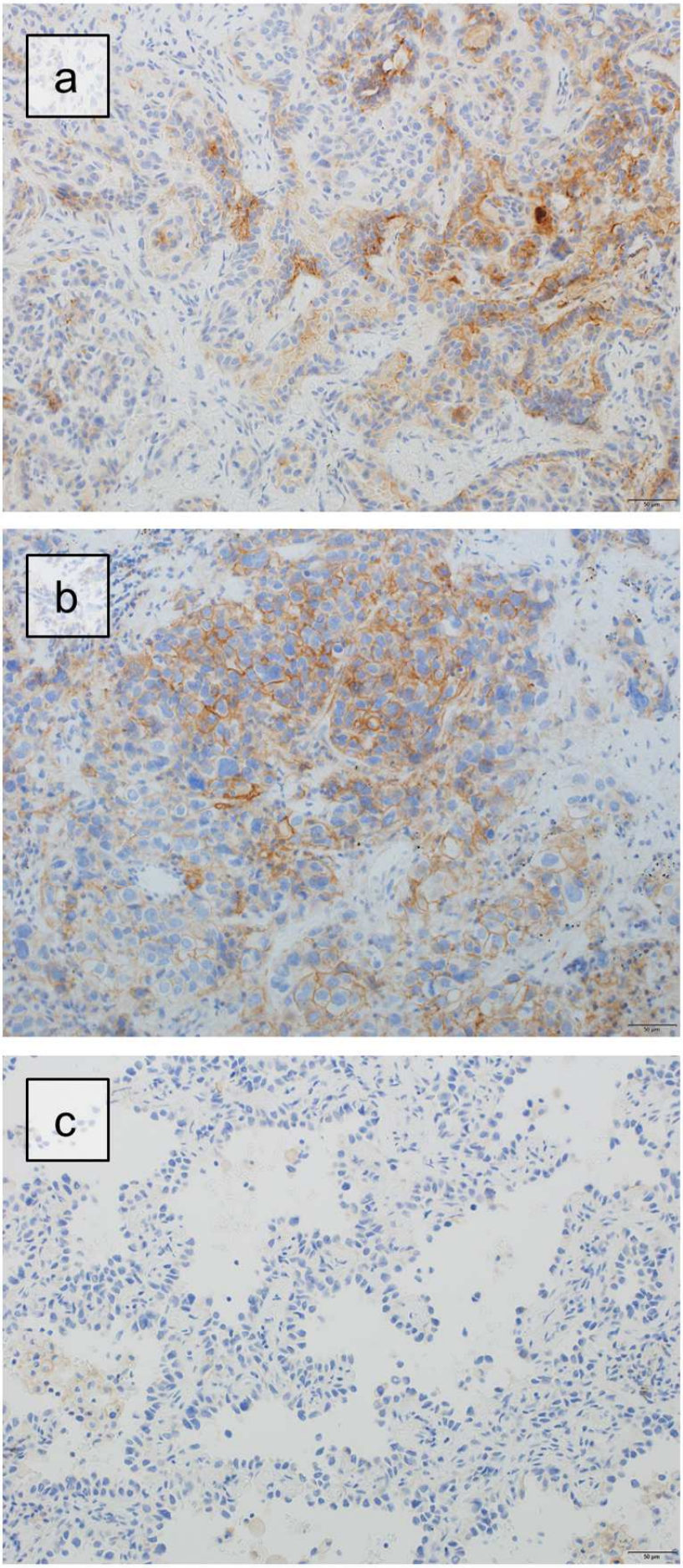


Figure2

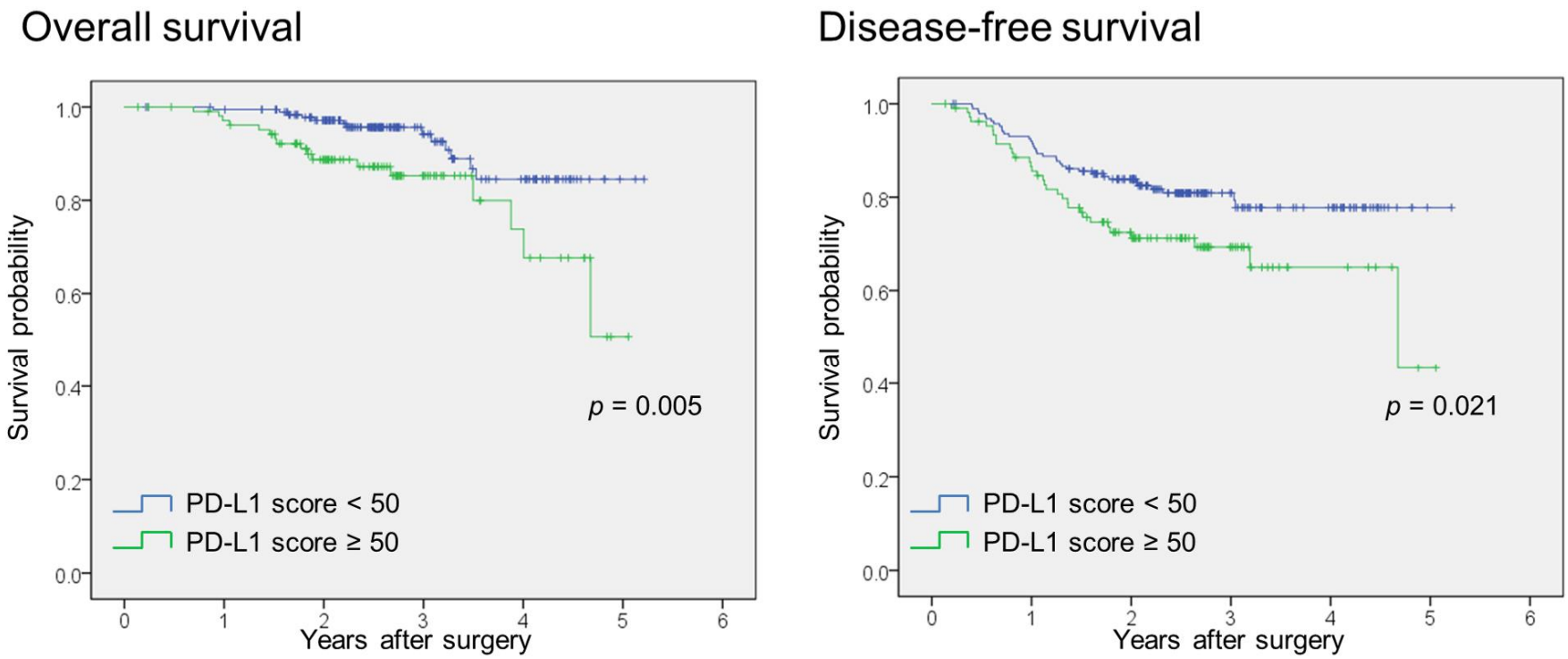
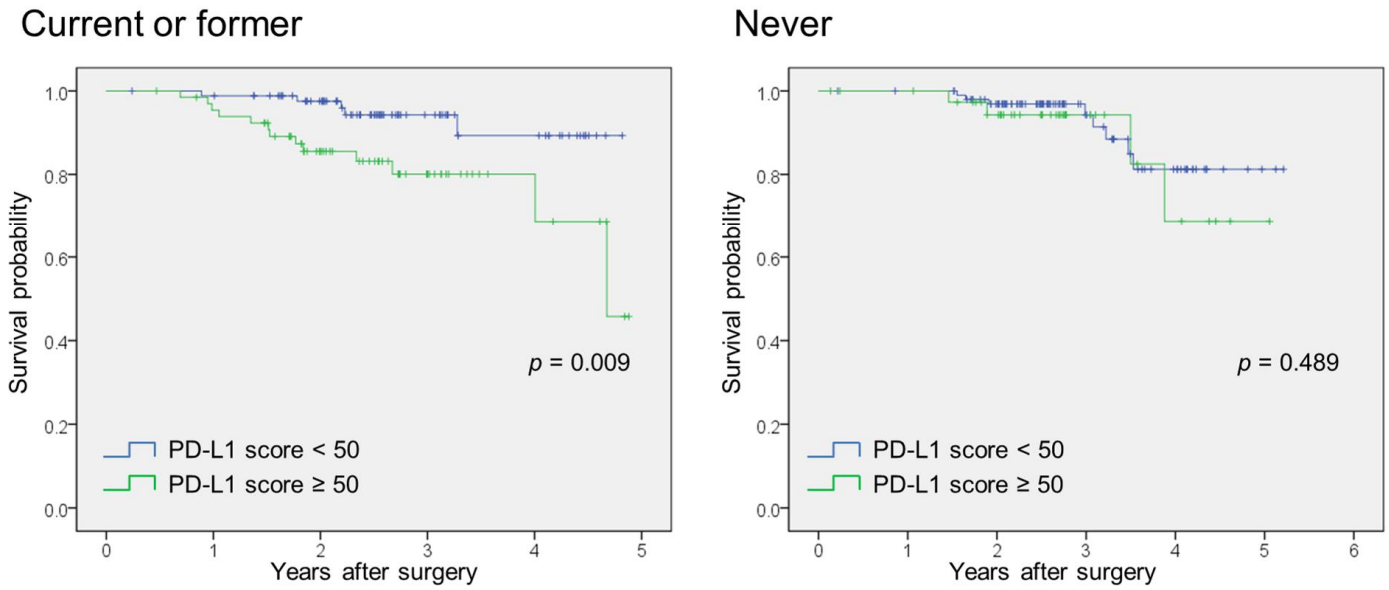
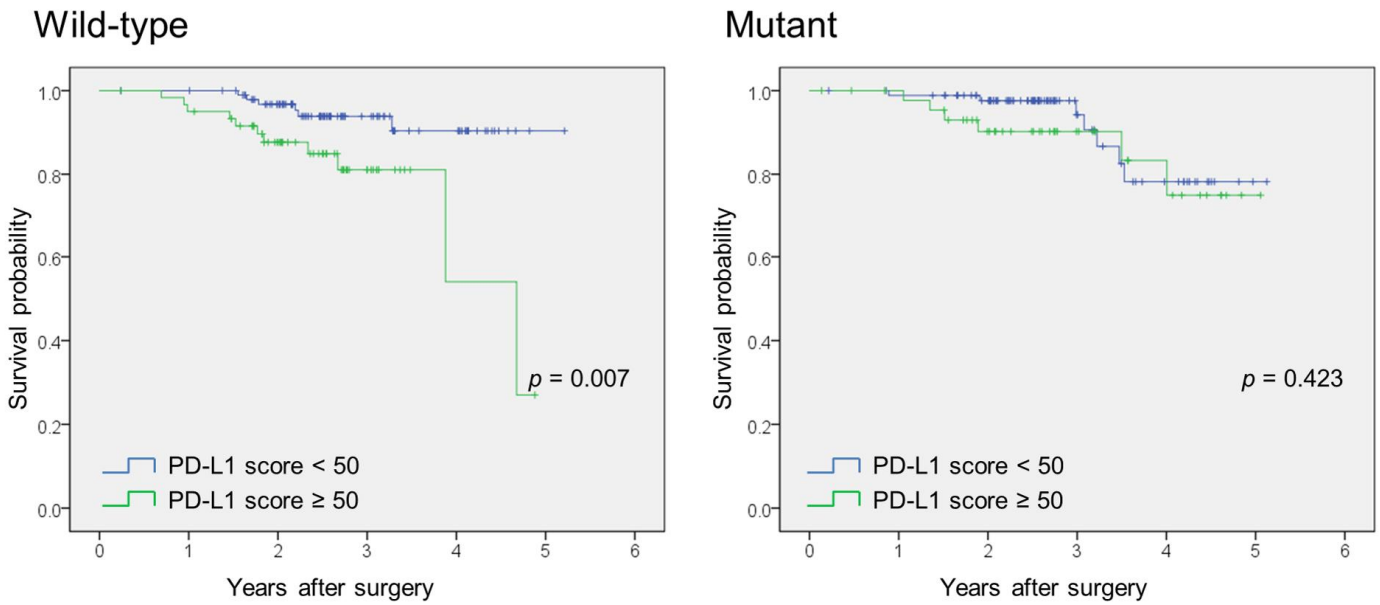

Overall survival
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.005
PD-L1 score < 50
PD-L1 score ≥ 50
0
1
2
3
4
5
6
Years after surgery
Disease-free survival
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.021
PD-L1 score < 50
PD-L1 score ≥ 50
0
1
2
3
4
5
6
Years after surgery

Figure3

a) Overall survival stratified by smoking history



b) Overall survival stratified by EGFR status



Supplementary Table 1 Smoking history and EGFR status

	EGFR status		
	Mutant	Wild-type	Total
Current/former smokers	50	104	154
Never smoked	86	56	142
Total	136	160	296

EGFR mutants include exon 19 deletion and L858R.