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Original Research

Indoleamine 2,3-dioxygenase 1 and programmed cell death-ligand 1 co-expression correlates with aggressive features in lung adenocarcinoma

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IDO1;
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Abstract ***Background:*** Indoleamine 2,3-dioxygenase 1 (IDO1) is an immunosuppressive effector, and its expression is associated with prognosis in several cancer types. Here, we investigated the relationship between IDO1 expression in lung adenocarcinoma and patient prognosis and clinicopathological features, including programmed cell death-ligand 1 (PD-L1) expression.

Materials and methods: In this study, surgically resected primary lung adenocarcinoma specimens from 427 patients were evaluated for IDO1 and PD-L1 expression by immunohistochemistry, and lung adenocarcinoma cell lines were evaluated for IDO1 and PD-L1 protein expression by enzyme-linked immunosorbent assay and flow cytometry and for messenger RNA levels by real-time reverse-transcriptase polymerase chain reaction analysis.

Results: IDO1 was expressed in 260 patients (60.9%) at 1% cut-off and 63 patients (14.8%) at 50% cut-off. Tissues from 145 patients (34.0%) were positive for PD-L1 using the cut-off of 1%. Multivariate analysis showed that ≥1% IDO1 positivity was significantly associated with higher tumour grade, vascular invasion and PD-L1 expression. IDO1 and PD-L1 proteins were co-expressed in 123 patients (28.8%), and co-expressing tumours exhibited significantly

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more malignant traits than those positive for one or neither protein. In multivariate analysis, co-expression of IDO1 and PD-L1 was significantly associated with shorter disease-free survival and overall survival. Both proteins were upregulated in lung adenocarcinoma cell lines by treatment with interferon-γ and transforming growth factor-β.
Conclusion: These results suggest that IDO1 and PD-L1 co-expression may define an aggressive form of lung adenocarcinoma.

1. Introduction

Lung cancer is a major health burden worldwide and is associated with high mortality [1]. Recent preclinical and clinical studies have considerably increased our understanding of the molecular pathogenesis of lung cancer and have facilitated the development of improved treatment strategies.

Targeting of immune checkpoint factors, such as the programmed cell death-1 (PD-1)/programmed cell death-ligand 1 (PD-L1) pathway, has emerged as a novel and promising therapeutic option [2]. Immune checkpoint inhibitors, such as the anti-PD-1 antibodies nivolumab and pembrolizumab and the anti-PD-L1 antibody atezolizumab, have shown survival benefits compared with conventional standard therapy in non-small-cell lung cancer (NSCLC) [3–5]. However, most patients who initially respond to these inhibitors acquire resistance, and several resistance mechanisms have been identified, including lack of tumour antigens or effective antigen presentation, impaired interferon-γ (IFN-γ) signalling, somatic Janus Kinase 1/2 mutations, impaired immune suppressive cells and/or immunoinhibitory cytokines, upregulation of other immune checkpoints and T-cell exhaustion [6–8]. Therefore, next generation immunotherapeutic drugs or combinations with cytotoxic chemotherapy and other molecularly targeted therapies should be explored to improve the response rate and to overcome resistance to immune checkpoint inhibitors.

Indoleamine 2,3-dioxygenase 1 (IDO1) catalyses the rate-limiting step in the kynurenine pathway that catabolizes tryptophan, an essential amino acid critical for cell survival, into a stable metabolite [9]. In the tumour microenvironment, IDO1 is expressed on antigen-presenting cells, such as macrophages, dendritic cells and tumour cells [9], whereas in normal settings, IDO1 is only expressed in tissues with large mucosal surface areas (lungs, gut and placenta) that experience chronic inflammation and lymphoid tissues [9,10]. IDO1 exerts its immunosuppressive effects in several ways, including induction of T cell dysfunction and apoptosis, promotion of naive T cell differentiation into regulatory T cells and impairment of natural killer cell function through the depletion of tryptophan and generation of kynurenine [11,12]. Aberrant expression of IDO1 has been shown to correlate with poor clinical outcome in breast, gastric, colorectal and ovarian cancers [13–16]. However, the clinical significance of IDO1 expression in lung adenocarcinoma has not been fully clarified. Furthermore, the association between IDO1 and immune checkpoint factors, such as PD-L1, remains unclear.

In this translational study, we investigated the association between IDO1 expression and clinicopathological factors and the prognostic value of IDO1 in patients with primary lung adenocarcinoma. We also evaluated the relationship between IDO1 and PD-L1 expression in these tumours. Finally, we examined IDO1 and PD-L1 expression and their modulation by cytokines in lung adenocarcinoma cell lines.

2. Materials and methods

2.1. Patients and samples

We performed a retrospective analysis of 427 patients who underwent surgical resection for primary lung adenocarcinoma between January 2003 and December 2012 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University. Patients with stage IV disease were excluded. Clinicopathological features, including age at surgery; sex; smoking history; tumour differentiation; pathological tumour, node and metastasis stage (seventh edition of the American Joint Committee on Cancer lung cancer staging system); pleural or lymphovascular invasion; histological subtype (World Health Organization Classification 2015); surgical procedure and epidermal growth factor receptor (*EGFR*) mutation status were recorded. The *EGFR* status of 250 specimens had previously been determined [17]. Clinical information and follow-up data were obtained from medical records. This study was approved by our Institutional Review Board (Kyushu University, IRB No. 29-318).

2.2. Immunohistochemical analysis

Formalin-fixed and paraffin-embedded (FFPE) tumour tissue sections were used for immunohistochemical

analysis. Staining for PD-L1 was performed as previously described [18] using a rabbit monoclonal anti-human PD-L1 antibody (clone SP142; Spring Bioscience, Tucson, AZ) at 1:100 dilution. For IDO1 staining, sections were cut (4 μm thickness), dewaxed with xylene and rehydrated through a graded series of ethanol solutions. Endogenous peroxidase activity was inhibited by incubation for 30 min with 3% H_2O_2 in methanol, and antigen retrieval was achieved by treatment with ethylene diamine tetra-acetic acid (pH 8.0) in a decloaking chamber at 110 °C for 15 min. The sections were then incubated with a 1:200 dilution of mouse anti-human IDO1 monoclonal antibody (clone UMAB126; Origene Technologies, Rockville, MD) at 4 °C overnight. Bound antibody was detected using a DAKO EnVision Detection System (Dako). Finally, the sections were incubated with 3,3′-diaminobenzidine, counter-stained with hematoxylin and mounted. We used sections from human placentas as positive controls for PD-L1 and IDO1 in this study.

The proportion of positive cells was independently estimated as the percentage of total carcinoma cells in whole sections by three investigators (K.T., K.K. and Y.K.) who were blinded to the patient clinical status. The final tumour proportion score (TPS) was reached by consensus. Specimens were considered negative for protein expression if PD-L1 tumour membrane staining was <1% or if IDO1 tumour cytoplasmic and membrane staining was <1%. Because little is known about the significance of IDO1 expression levels, we evaluated the relationships between clinicopathological features and IDO1/PD-L1 expression levels using the cut-off values both 1% and 50% in this study.

2.3. Cell culture and cytokine treatment

See the Supplementary Text, available at *European Journal of Cancer* online.

2.4. RNA extraction and real-time reverse-transcriptase polymerase chain reaction analysis

See the Supplementary Text, available at *European Journal of Cancer* online.

2.5. Enzyme-linked immunosorbent assay

See the Supplementary Text, available at *European Journal of Cancer* online.

2.6. Flow cytometric analysis

See the Supplementary Text, available at *European Journal of Cancer* online.

2.7. Statistical analysis

See the Supplementary Text, available at *European Journal of Cancer* online.

3. Results

3.1. Association between IDO1 expression and clinicopathological factors in patients with primary lung adenocarcinoma

A total of 427 patients with primary lung adenocarcinoma who underwent surgical resection were included in this study (Supplementary Table 1). Of these, 211 (49.4%) were male, and 205 (48.0%) were smokers. The median age was 69 years (range: 29–85). The *EGFR* mutation status was available for 250 patients, of whom 118 (47.2%) harbored mutant *EGFR*: 43 (36.4%) with exon 19 deletions, 69 (58.5%) with exon 21 L858R point mutations and 6 (5.1%) with other minor mutations. PD-L1 expression was positive (>1%) in specimens from 145 patients (34.0%).

Fig. 1Ashows immunohistochemical staining of IDO1 in a section of human placenta, which was used as a positive control. Strong cytoplasmic and membrane staining of the endothelial cells is evident. Immunohistochemical staining of IDO1 was detected in both the cytoplasm and membrane of cancer cells (Fig. 1B). Representative images with IDO1 staining TPS of <1%, 1–50% and ≥50% are shown in Fig. 1C–E, respectively. The associations between IDO1 expression and clinicopathological factors are described in Table 1. Using 1% and 50% as cut-off values, 260 (60.9%) and 63 (14.8%) patients were positive for IDO1 expression, respectively. Multivariate analysis showed that IDO1 positivity (1% cut-off) was significantly associated with higher tumour grade, vascular invasion and PD-L1 expression (Table 2).

3.2. Univariate and multivariate analyses of disease-free survival and overall survival in patients with primary lung adenocarcinoma according to IDO1 expression

We next analysed survival analysis according to IDO1 expression using the Kaplan–Meier method. At the end of data collection for this study, the median follow-up time was 61.6 months (range: 0.6–145.3). Shorter disease-free survival (DFS) was significantly associated with IDO1 positivity at both 1% and 50% cut-off values (log-rank test: $P = 0.0002$ and $P = 0.0162$, respectively; Fig. 2A and C), whereas overall survival (OS) was significantly associated with IDO1 at the 1% but not the 50% cut-off value (log-rank test: $P = 0.0018$ and $P = 0.2809$, respectively; Fig. 2B and D). Based on these

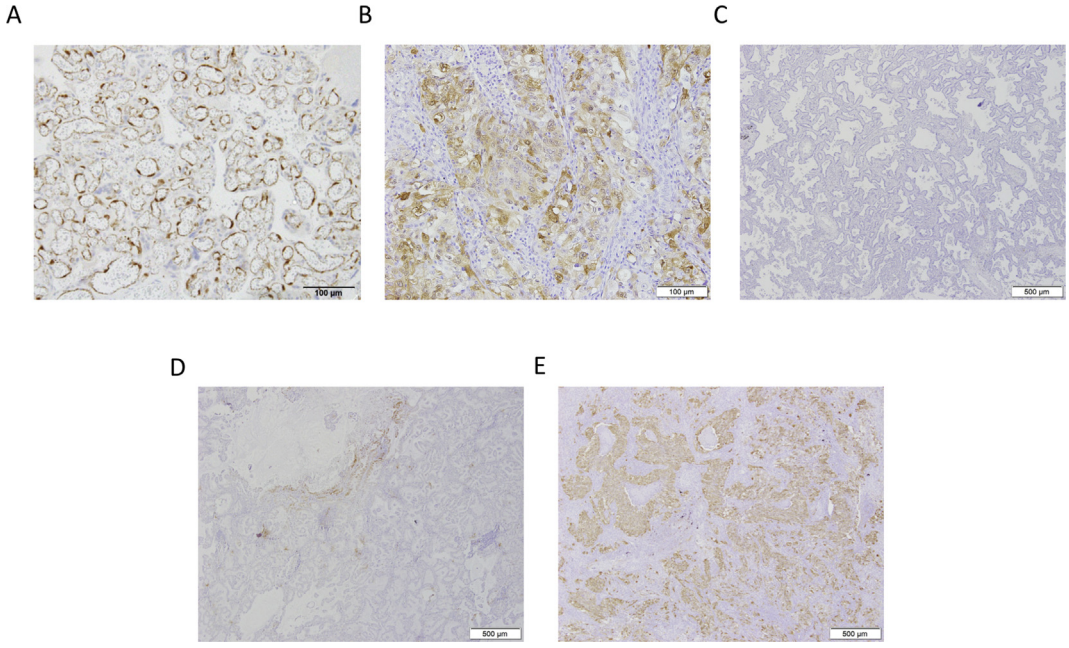

Fig. 1. Representative images of immunohistochemical staining of IDO1 in human placental tissue and surgically resected specimens from patients with primary lung adenocarcinoma. (A) Human placental tissue showing strong cytoplasmic and membrane staining in endothelial cells. Scale bar: 100 μm. (B) Lung adenocarcinoma showing positive cytoplasmic and membrane staining. Scale bar: 100 μm. (C–E) Typical IDO1 staining of lung adenocarcinoma at TPS of <1% (C), 1–50% (D), and ≥50% (E). Scale bars: 500 μm. IDO1, indoleamine 2,3-dioxygenase 1; TPS, tumour proportion score.

Table 1
Association between IDO1 expression and patient clinicopathological factors.

Factors		*N*	1% cut-off			50% cut-off		
			IDO1, *N* (%)		*P* value	IDO1, *N* (%)		*P* value
			Negative	Positive		Negative	Positive	
Age (years)	<70	227	86 (51.5)	141 (54.2)	0.6197	198 (54.4)	29 (46.0)	0.2225
	≥70	200	81 (48.5)	119 (45.8)		166 (45.6)	34 (54.0)	
Sex	Male	211	79 (47.3)	132 (50.8)	0.4896	174 (47.8)	37 (58.7)	0.1331
	Female	216	88 (52.7)	128 (49.2)		190 (52.2)	26 (41.3)	
Smoking status	Never smoker	222	90 (53.9)	132 (50.8)	0.5524	198 (54.4)	24 (38.1)	0.02
	Smoker	205	77 (46.1)	128 (49.2)		166 (45.6)	39 (61.9)	
T	T1	252	109 (65.3)	143 (55.0)	0.0436	215 (59.1)	37 (58.7)	1
	≥T2	175	58 (34.7)	117 (45.0)		149 (40.9)	26 (41.3)	
N	N0	347	151 (90.4)	196 (75.4)	<0.0001	299 (82.1)	48 (76.2)	0.2937
	≥N1	80	16 (9.6)	64 (24.6)		65 (17.9)	15 (23.8)	
Stage	I	315	137 (82.0)	178 (68.5)	0.0022	272 (74.7)	43 (68.3)	0.2811
	II/III	112	30 (18.0)	82 (31.5)		92 (25.3)	20 (31.7)	
Grade	G1	203	116 (69.5)	87 (33.5)	<0.0001	192 (52.8)	11 (17.5)	<0.0001
	≥G2	224	51 (30.5)	173 (66.5)		172 (47.2)	52 (82.5)	
Pleural invasion	Absent	331	140 (83.8)	191 (73.5)	0.0127	288 (79.1)	43 (68.3)	0.0713
	Present	96	27 (16.2)	69 (26.5)		76 (20.9)	20 (31.7)	
Lymphatic invasion	Absent	366	152 (91.0)	214 (82.3)	0.0154	313 (86.0)	53 (84.1)	0.6975
	Present	61	15 (9.0)	46 (17.7)		51 (14.0)	10 (15.9)	
Vascular invasion	Absent	307	146 (87.4)	161 (61.9)	<0.0001	272 (74.7)	35 (55.6)	0.0036
	Present	120	21 (12.6)	99 (38.1)		92 (25.3)	28 (44.4)	
Histological subtype	Micropapillary/solid	26	4 (2.4)	22 (8.5)	0.0117	15 (4.1)	11 (17.5)	0.0004
	Others	401	163 (97.6)	238 (91.5)		349 (95.9)	52 (82.5)	
Surgical procedure	≥Lobectomy	327	118 (70.7)	209 (80.4)	0.0259	277 (76.1)	50 (79.4)	0.6319
	Sublobar resection	100	49 (29.3)	51 (19.6)		87 (23.9)	13 (20.6)	
EGFR[a]	Wild type	132	48 (47.1)	84 (56.8)	0.1564	108 (50.7)	24 (64.9)	0.1529
	Mutant	118	54 (52.9)	64 (43.2)		105 (49.3)	13 (35.1)	
PD-L1	Negative	282	145 (86.8)	137 (52.7)	<0.0001	256 (70.3)	26 (41.3)	<0.0001
	Positive	145	22 (13.2)	123 (47.3)		108 (29.7)	37 (58.7)	

IDO1, indoleamine 2,3-dioxygenase 1; *EGFR*, epidermal growth factor receptor gene; PD-L1, programmed cell death-ligand 1.
[a] Cases for which data were available.

Table 2
Univariate and multivariate analyses of the relationship between IDO1 expression and other patient clinicopathological factors.

Factors		1% cut-off				50% cut-off			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (years)	≥70/< 70	0.90 (0.61–1.32)	0.5807			1.40 (0.82–2.40)	0.2198		
Sex	Male/female	1.15 (0.78–1.70)	0.4847			1.55 (0.91–2.69)	0.1085		
Smoking status	Smoker/never smoker	1.13 (0.77–1.67)	0.5284			1.94 (1.13–3.39)	0.0165		
Stage	≥II/I	2.10 (1.32–3.42)	0.0015			1.38 (0.76–2.43)	0.2891		
Grade	≥G2/G1	4.52 (2.99–6.91)	<0.0001	2.35 (1.43–3.87)	0.0007	5.28 (2.77–10.96)	<0.0001	4.01 (2.03–8.56)	<0.0001
Pleural invasion	Present/absent	1.87 (1.15–3.11)	0.0109			1.76 (0.96–3.14)	0.0651		
Lymphatic invasion	Present/absent	2.18 (1.20–4.17)	0.0099			1.16 (0.53–2.34)	0.7001		
Vascular invasion	Present/absent	4.28 (2.58–7.36)	<0.0001	2.15 (1.18–4.01)	0.0121	2.37 (1.36–4.10)	0.0026		
Histological subtype	Micropapillary, solid/others	3.77 (1.41–13.06)	0.0065			4.92 (2.10–11.25)	0.0004		
Surgical procedure	≥Lobectomy/ sublobar resection	1.70 (1.08–2.68)	0.0215			1.21 (0.64–2.41)	0.5673		
EGFR[a]	Wild type/mutant	1.48 (0.89–2.46)	0.1311			1.79 (0.88–3.81)	0.1084		
PD-L1	Positive/negative	5.92 (3.61–10.07)	<0.0001	4.05 (2.40–7.06)	<0.0001	3.37 (1.96–5.90)	<0.0001	2.20 (1.23–3.98)	0.0076

IDO1, indoleamine 2,3-dioxygenase 1; *EGFR*, epidermal growth factor receptor gene; PD-L1, programmed cell death-ligand 1; OR, odds ratio; CI, confidence interval.
[a] Cases for which data were available.

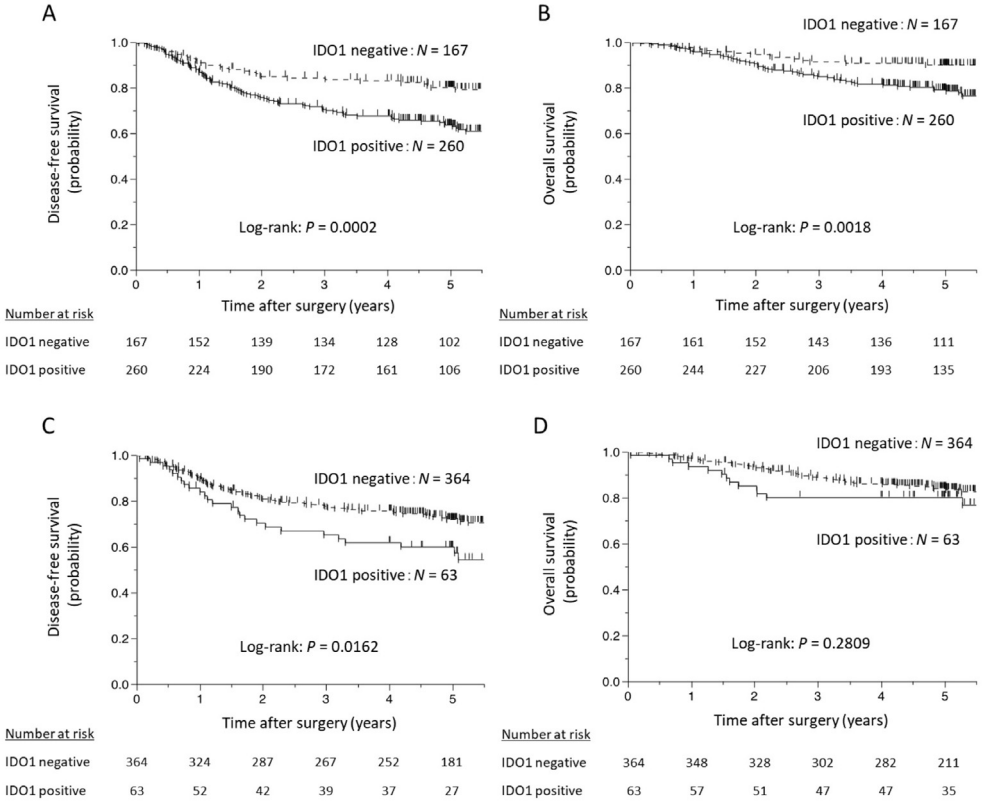

Fig. 2. Kaplan–Meier curves showing survival of patients with primary lung adenocarcinoma according to IDO1 expression. (A) Disease-free survival and (B) overall survival according to IDO1 expression status determined by the 1% cut-off value. (C) Disease-free survival and (D) overall survival according to IDO1 expression status, as determined by the 50% cut-off value. IDO1, indoleamine 2,3-dioxygenase 1; TPS, tumour proportion score.

results, we used the 1% cut-off value for multivariate analysis of survival; however, IDO1 positivity did not remain a predictor of either DFS or OS (Cox proportional hazards regression model; data not shown).

3.3. Co-expression of IDO1 and PD-L1 in primary lung adenocarcinoma and survival analysis

We evaluated the association between IDO1 and PD-L1 expression in primary lung adenocarcinoma and their relationship to survival. At 1% cut-off, a significant correlation was detected between IDO1 and PD-L1 expression (Table 1). Furthermore, all patients with strong PD-L1 expression (TPS $\geq$ 50%) were positive for IDO1 (Supplementary Table 2). We further conducted a combinatory analysis of IDO1 and PD-L1, with the cut-off values for both being 1%. The associations between clinicopathological factors and IDO1/PD-L1 co-expression are shown in Supplementary Table 3. IDO1- and PD-L1-expressing adenocarcinomas were associated with more malignant traits than tumours expressing one or neither protein, and co-expression was also significantly associated with smoking and expression of wild-type *EGFR*.

DFS and OS were analysed in patients categorised as IDO1−/PD-L1−, IDO1−/PD-L1+, IDO1+/PD-L1− and IDO1+/PD-L1+. Significant differences in DFS and OS were noted among the four groups, as shown in Fig. 3A and B (log-rank test: $P < 0.0001$ for both DFS and OS).

Co-expression of IDO1 and PD-L1 was observed in samples from 123 patients (28.8%). Co-expression was significantly associated with shorter DFS and OS using a Cox proportional hazards regression model (Table 3). In multivariate analysis, co-expression of IDO1 and PD-L1 remained an independent predictor of DFS and OS (HR = 1.57, $P = 0.0210$ and HR = 2.58, $P < 0.0001$, respectively).

3.4. IDO1 and PD-L1 expression in lung adenocarcinoma cell lines

To investigate IDO1 and PD-L1 expression further, we evaluated 10 lung adenocarcinoma cell lines. Supplementary Table 4 provides a summary of the IDO1 and PD-L1 protein and messenger RNA expression levels in the cell lines. We did not detect a relationship between IDO1 expression and either oncogene status or PD-L1 expression. Next, we compared protein expression in untreated control cells and IFN-γ- or transforming growth factor-β (TGF-β)-treated cells. As shown in Supplementary Fig. 1, expression of both IDO1 and PD-L1 were elevated by IFN-γ and TGF-β to levels significantly higher than those in untreated control cells.

4. Discussion

We detected IDO1 expression in resected lung adenocarcinoma from 60.9% to 14.8% of patients using a cut-off of 1% and 50%, respectively. Previous studies demonstrated that IDO1 was expressed in tumour tissue from 40 to 79% of NSCLC patients [10,19]. Because immunohistochemical evaluation of IDO1 in lung adenocarcinoma is not well established, we analysed the data based on both 1% and 50% positivity cut-offs. At 1%, the proportion of IDO1-positive tumours in our study was similar to that previously reported, and this expression cut-off also predicted postoperative prognosis more sensitively than did the 50% cut-off value. Therefore, a 1% cut-off value was used for further analyses. We do not know the reason for this result, but it

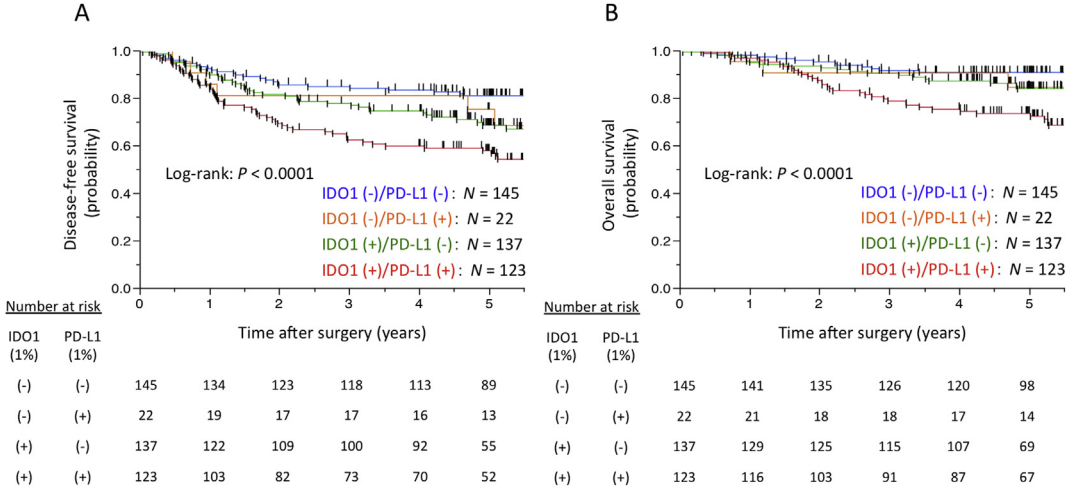

Fig. 3. Kaplan–Meier curves showing survival of patients with primary lung adenocarcinoma according to IDO1 and PD-L1 expression. We adopted cut-off values of 1% for IDO1 and PD-L1 expression in this analysis. (A) Disease-free survival and (B) overall survival of patients expressing the indicated combinations of IDO1 and PD-L1. IDO1, indoleamine 2,3-dioxygenase 1; PD-L1, programmed cell death-ligand 1.

Table 3
Univariate and multivariate analyses of DFS and OS in all patients.

Factors		DFS				OS			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (years)	≥70/< 70	1.45 (1.03–2.03)	0.0318	1.59 (1.13–2.23)	0.0076	2.64 (1.69–4.24)	<0.0001	3.35 (2.12–5.42)	<0.0001
Sex	Male/female	1.91 (1.35–2.71)	0.0002			2.31 (1.48–3.70)	0.0002	2.21 (1.40–3.55)	0.0005
Smoking status	Smoker/never smoker	1.47 (1.04–2.06)	0.0273			1.71 (1.10–2.67)	0.0164		
Stage	≥II/I	5.64 (4.01–7.97)	<0.0001	3.48 (2.40–5.07)	<0.0001	4.49 (2.91–6.99)	<0.0001	3.13 (1.97–5.01)	<0.0001
Grade	≥G2/G1	3.94 (2.67–5.99)	<0.0001	1.85 (1.18–2.98)	0.0075	3.75 (2.27–6.51)	<0.0001		
Pleural invasion	Present/absent	3.39 (2.38–4.78)	<0.0001			3.59 (2.31–5.55)	<0.0001		
Lymphatic invasion	Present/Absent	4.87 (3.37–6.95)	<0.0001	2.84 (1.88–4.25)	<0.0001	4.28 (2.69–6.69)	<0.0001	3.42 (2.07–5.53)	<0.0001
Vascular invasion	Present/Absent	3.09 (2.20–4.35)	<0.0001			3.46 (2.24–5.36)	<0.0001		
Histological subtype	Micropapillary, solid/Others	1.99 (1.10–3.35)	0.0255			1.24 (0.48–2.62)	0.6238		
Surgical procedure	≥Lobectomy/ Sublobar resection	1.58 (1.03–2.54)	0.0352			1.67 (0.96–3.16)	0.0729		
EGFR[a]	Wild-type/Mutant	1.74 (1.09–2.82)	0.019			2.02 (1.09–3.94)	0.0246		
IDO1 and PD-L1[b]	Co-expression/Others	2.07 (1.46–2.90)	<0.0001	1.57 (1.07–2.29)	0.0210	2.79 (1.81–4.31)	<0.0001	2.58 (1.65–4.04)	<0.0001

DFS, disease-free survival; OS, overall survival; *EGFR*, epidermal growth factor receptor gene; IDO1, indoleamine 2,3-dioxygenase 1; PD-L1, programmed cell death-ligand 1; HR, hazard ratio; CI, confidence interval.
[a] Cases for which data were available.
[b] The cut-off values for IDO1 and PD-L1 were 1%.

may mean that even minimal IDO1 expression is related to poor prognosis. In this study, only 63 (14.8%) patients were positive for IDO1 at the 50% cut-off value, and such a low positive rate may be one of the reasons for the observed associations with prognosis.

We demonstrated that patients with IDO1-expressing lung adenocarcinoma exhibited shorter DFS and OS than those lacking IDO1. Some studies have previously evaluated the prognostic role of IDO1 in NSCLC [19,20]. In a recent report, Schalper et al. [19] investigated two cohorts of 202 and 350 patients with NSCLC, including adenocarcinoma, squamous cell carcinoma and other subtypes. They found that the relationship between survival and IDO1 expression was inconsistent: IDO1 expression was associated with a favourable prognosis in one cohort but not in the other [19]. This discrepancy may be due to differences in clinicopathological factors, such as smoking status and histological subtype, between the two cohorts.

In this study, we evaluated the expression of IDO1 and PD-L1 proteins in primary lung adenocarcinoma. Co-expression of IDO1 and PD-L1 was found in 123 patients (28.8%), which is considerably higher than the ~10% of NSCLC patients co-expressing IDO1 and PD-L1 in another study [19]. Parra et al. [21] recently reported the expression of IDO1, PD-L1 and other immune checkpoint markers in tissue microarray specimens from surgically resected NSCLC patients by immunohistochemistry (adenocarcinoma: $N = 123$, squamous cell carcinoma: $N = 61$). They detected IDO1 and PD-L1 co-expression in 37% of the lung adenocarcinoma patients. These differences could be due to the use of distinct anti-IDO1 and PD-L1 antibodies and/or other features of the immunohistochemical assay. In the earlier study [19], Schalper et al. evaluated the expression of IDO1 and PD-L1 using quantitative immunofluorescence on tissue microarray sections, whereas we performed immunohistochemical staining of FFPE tumour tissue sections from surgically resected lung adenocarcinomas. Moreover, Parra et al. [21] evaluated IDO1 and PD-L1 expression using immunohistochemistry on tissue microarray specimens. Expression of both IDO1 and PD-L1 is induced on tumour cells and immune cells by local inflammatory signals such as IFN-γ and TGF-β [2,22,23]. In this study, we demonstrated that IDO1 and PD-L1 were both upregulated in lung adenocarcinoma cell lines following treatment with IFN-γ and TGF-β, confirming that the tumour microenvironment may influence IDO1 and PD-L1 expression. Therefore, the differences in IDO1/PD-L1 co-expression rates between our study and those of Schalper et al. [19] and Parra et al. [21] may also be due to heterogeneity in the tumour specimens. Moreover, variations in patient characteristics, such as smoking status and tumour histology, may have contributed to the different findings. In the study by Schalper et al. [19],

the NSCLC cohort included patients with adenocarcinoma, squamous adenocarcinoma and other tumour types, and the proportion of smokers was high (80–91%). In the study by Parra et al. [21], the NSCLC cohort included patients with adenocarcinoma and squamous adenocarcinoma, and the proportion of smokers was also high (88% of the patients with adenocarcinoma and 99% of the patients with squamous cell carcinoma). In contrast, our study investigated only adenocarcinoma patients, and only half of our patients were smokers. We showed that IDO1/PD-L1 co-expression was significantly associated with smoking and with wild-type *EGFR* expression in this study. Lung squamous cell carcinoma, which we did not study here, is known to have a close etiological relationship with smoking; thus, the smoking status and histology may strongly affect the co-expression rate [24]. We plan to evaluate IDO1 and PD-L1 expression in lung squamous cell carcinoma in a future study.

We showed that IDO1 and PD-L1 co-expression in lung adenocarcinoma was significantly associated with more malignant traits and that co-expression of IDO1 and PD-L1 was more significantly associated with poor prognosis compared with expression of one or neither protein and was an independent predictor of prognosis. The negative synergistic effect of the two proteins on prognosis suggests that combination therapy targeting both IDO1 and PD-L1 may improve the clinical outcome of lung adenocarcinoma patients through reactivation of antitumour immunity. Many preclinical studies have demonstrated the antitumour efficacy of IDO1 inhibition [25–27]. Moreover, the combination therapy of IDO1 inhibitor and PD-1/PD-L1 inhibitor is attracting a lot of attention now. Indeed, the IDO1 inhibitor epacadostat in combination with the PD-L1 inhibitor atezolizumab is being evaluated in a phase I study for NSCLC [28]. In addition, several clinical trials have shown that the combination therapy of epacadostat and the PD-1 inhibitor pembrolizumab might be one of the novel and effective treatment options in patients with solid tumours, such as breast cancer, ovarian cancer, urothelial carcinoma, renal cell carcinoma and NSCLC [29–32]. Previous studies showed that high IDO1 expression in tumour cells was associated with lower CD3+ and CD8+ T lymphocyte infiltration in some cancer types [33,34]. Moreover, IDO1 inhibition was shown to induce tumour infiltration by CD3+ and CD8+ T lymphocytes *in vivo*, suggesting that combination therapy with IDO1 and PD-1/PD-L1 inhibitors might improve treatment efficacy compared with single-agent immunotherapy [35]. In *in vivo* studies, combination therapies targeting IDO1 and PD-1/PD-L1 synergistically led to the enhanced infiltration of tumour-specific effector T cells and a marked increase in the effector-to-regulatory T cell ratios in tumours, which demonstrated the synergistic effect of IDO1 and PD-L1 blockade [36]. It is unclear whether the therapeutic effect of IDO1 inhibitors depends on the degree of IDO1 expression on tumour cells; however, our findings indicate that IDO1 and PD-L1 co-expression could be a predictive maker and both are therapeutic targets.

There are several limitations associated with the present study. First, it was a single institutional retrospective study and not a trial-based correlative study; thus, the possibility of bias cannot be excluded. Validation cohort studies should be conducted to confirm our results. Second, we conducted PD-L1 immunohistochemistry using only one antibody. Several recent studies showed that positive expression of PD-L1 was detected at a lower rate with the SP142 antibody used here than with other antibodies, such as 28-8, 22C3, and SP263 [37–40]. In a future study, we will evaluate PD-L1 expression using other antibodies. Third, there are no definitive guidelines for antibody use or quantifying IDO1 expression in lung adenocarcinoma. We used UMAB126 and set the cut-off values for positivity as 1% and 50% staining of the cytoplasm and membrane of cancer cells. However, this antibody has not been evaluated in a clinical setting, and a previous study used a different scoring system based on the intensity and extent of positive staining [20]. Thus, the predictive value of IDO1 expression is likely to be most useful if a standardised quantitative assay is established. Fourth, we evaluated patients with surgically resected lung adenocarcinoma, and most of the patients were diagnosed with early cancer. In future studies, analysis of IDO1 and PD-L1 expression in patients with unresectable advanced or recurrent disease will shed light on the utility of these markers for identifying patients who might benefit from single-agent or combination immunotherapy. In this study, we evaluated IDO1 and PD-L1 expression in surgically resected primary lung adenocarcinomas, most of which were diagnosed as early-staged cancer, in a cohort of Japanese patients. There is the higher proportion of never-smokers and *EGFR* mutations among Japanese patients compared with Caucasian patients [41,42]. Moreover, the SP142 antibody shows lower rates of PD-L1 expression compared with other antibodies. Therefore, we think that only 34% of the whole group was positive (at >1%) for PD-L1 in this study, meaning it will be necessary to evaluate these factors in a cohort of Caucasian patients.

In conclusion, we demonstrated that IDO1 expression in lung adenocarcinoma was associated with poor prognosis, and that co-expression with PD-L1 was an independent predictor of shorter DFS and OS.

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Conflict of interest statement

The authors declare no conflicts of interest in association with the present study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejca.2018.06.020.

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