JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 55 (1): 25–35, 2022
doi: 10.1267/ahc.21-00075

Regular Article

Immunolocalization of CD80 and CD86 in Non-Small Cell Lung Carcinoma: CD80 as a Potent Prognostic Factor

Takashi Sato[1,2], Kiyoshi Takagi[2], Mitsunori Higuchi[3], Hiroko Abe[1], Michie Kojimahara[1], Miho Sagawa[1], Megumi Tanaki[1], Yasuhiro Miki[4], Takashi Suzuki[2] and Hiroshi Hojo[5]

[1]Department of Clinical Laboratory, Aizu Medical Center, Fukushima Medical University, Aizuwakamatsu, Japan, [2]Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, Sendai, Japan, [3]Department of Thoracic Surgery, Aizu Medical Center, Fukushima Medical University, Aizuwakamatsu, Japan, [4]Department of Disaster Obstetrics and Gynecology, International Research Institute of Disaster Science, Tohoku University, Sendai, Japan and [5]Department of Pathology, Aizu Medical Center, Fukushima Medical University, Aizuwakamatsu, Japan

Received September 3, 2021; accepted December 10, 2021; published online February 11, 2022

It has been demonstrated that tumor cells express programed cell death protein 1 (PD-L1) to escape T lymphocytes that express programed cell protein 1 (PD-1), and PD-1/PD-L1 immune checkpoint inhibitors have been regarded in lung cancer patients. CD80 and CD86 are members of B7 superfamily which regulates T lymphocyte activation and tolerance. However, immunolocalization of CD80 and CD86 has not been examined in the lung carcinoma tissues and their clinical significance remains unknown. Therefore, to clarify clinical significance of CD80 and CD86, we immunolocalized these in 75 non-small cell lung carcinomas (NSCLC) in this study. Immunoreactivities of CD80 and CD86 were mainly detected in tumor-infiltrating macrophages. Immunohistochemical CD80 status was high in 56% of NSCLC, and it was positively associated with stage, pathological T factor, distant metastasis, histological type and PD-L1 status. Moreover, multivariate analysis turned out that the CD80 status was an independent worse prognostic factor. CD86 status was high in 53% of the cases, but it was not significantly associated with any clinicopathological parameters. These findings suggest that CD80 is a potent worse prognostic factor possibly in association with escape from immune attack in NSCLC.

Key words: CD80, CD86, immunohistochemistry, lung cancer, PD-L1

I. Introduction

Lung cancer is one of the most common fatal malignancies in worldwide [24], and the incidence is increasing. Histologically, lung carcinoma is subclassified into small (approximately 20%) and non-small cell lung carcinoma (NSCLC). NSCLC accounts for approximately 80% of lung carcinomas and is composed of heterogenous groups including adenocarcinoma and squamous cell carcinoma. NSCLC generally responds poorly to chemotherapy compared to small cell carcinoma [32], and various molecular targeted therapeutic agents have been developed [22]. Recently, it has been demonstrated that tumor cells express programed cell death protein 1 (PD-L1) to escape T lymphocytes that express programed cell protein 1 (PD-1) [17], and PD-1/PD-L1 immune checkpoint inhibitors have been regarded as a promising therapeutic strategy for lung cancer patients [5].

B7-1 (CD80) and B7-2 (CD86) are members of B7 superfamily which regulates T cell activation and tolerance [18], as well as PD-L1. CD80/86 molecules on the surface

Correspondence to: Takashi Suzuki, MD, PhD., Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, 2–1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980–8575, Japan.
E-mail: t-suzuki@patholo2.med.tohoku.ac.jp

Table 1. *Procedures of automatic immunostaning for CD80, CD86 and PD-L1 in this study*

	CD80	CD86	PD-L1
Platform	Ventana Benchmark XT[a]	Bond III platform[b]	Ventana Benchmark XT[a]
Primary antibody (clone)	EPR1157(2)[c]	EP1158-37[c]	SP142[a]
Detection kit	OptiView DAB IHC Detection Kit[a]	BOND Polymer Refine Detection[b]	OptiView DAB IHC Detection Kit[a] and OptiView Amplification Kit[a]
Antigen retrieval	CC1[a] for 64 min	BOND Epitope Retrieval Solution 2[b] for 20 min	CC1[a] for 48 min
Dilution of primary antibody	1:500	1:100	Diluted antibody
Reaction time to primary antibody	32 min	15 min	16 min
Reaction time to detection kit	OptiView DAB for 8 min	DAB solution for 10 min	OptiView DAB for 8 min
	OptiView Peroxidase Inhibitor for 4 min	Peroxide Block for 5 min	OptiView Peroxidase Inhibitor for 4 min
	OptiView HQ Universal Linker for 8 min	Post Primary for 8 min	OptiView HQ Universal Linker for 8 min
	OptiView HRP Multimer for 8 min	Polymer for 8 min	OptiView HRP Multimer for 8 min
			OptiView Amplifier and OptiView Amplification H_2O_2 for 8 min
			OptiView Amplification Multimer for 8 min

[a]; Roche Diagnostics Japan (Tokyo, Japan), [b]; Leica Biosystems Japan (Tokyo, Japan), [c]; Abcam (Cambridge, UK).
DAB; 3,3'-diaminobenzidine

of antigen presenting cells bind to cytotoxic T cell antigen 4 (CTLA-4) on the surface of T cells, with much higher affinity to CD28, and suppress T cell activation [29]. CD80 also binds PD-L1 and inhibits T cell responses [1]. Therefore, it is suggestive that CD80/86 molecules play important roles in the regulation of immune microenvironment in lung carcinoma tissues. Expression of CD80 and/or CD86 molecules has been reported in hematologic malignancies [9] and several solid tumors such as glioma [3], gastric carcinoma [14] and pancreatic carcinoma [27]. However, immunolocalization of CD80 and CD86 has not been examined the lung carcinoma to the best of our knowledge. Therefore, in this study, we performed immunohistochemistry for CD80 and CD86 as well as PD-L1 in 75 NSCLC to clarify their clinicopathological significance.

II. Materials and Methods

Patients and tissues

75 specimens of primary NSCLC were obtained from Japanese patients (age range; 43–90 years) who underwent surgical or endoscopic treatment. These cases were obtained from 2016 to 2018 from Fukushima Medical University Aizu Medical Center (Aizuwakamatsu, Japan), and the specimens were fixed in 10% formalin and embedded in paraffin wax. Among the 75 patients, 28 patients received adjuvant chemotherapy after the surgical or endoscopic treatment. The intratumoral mononuclear infiltration was histologically evaluated as low (no areas or scattered small foci) or high (scattered large foci, numerous large or broad areas with pertinent changes) according to a previous report [25]. Clinical outcome of the patients was evaluated by overall survival, which was defined as the time from surgery or endoscopy to death. The mean follow-up time was 983 days (range; 21–1,504 days) in this study. The research protocol was approved by the Ethics Committee at the Fukushima Medical University.

Immunohistochemistry

Rabbit monoclonal antibodies for CD80 (ab269587, clone EPR1157(2)), CD86 (ab134120, clone EP1158-37) and PD-L1 (SP142) were purchased from Abcam (Cambridge, UK), Abcam and Roche Diagnostics Japan (Tokyo, Japan), respectively. Immunostaining for CD80, CD86 and PD-L1 antibodies was automatically performed using Ventana Benchmark XT platform (Roche Diagnostics Japan), Bond III platform (Leica Biosystems Japan, Tokyo, Japan) and Ventana Benchmark XT platform (Table 1). The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution with hematoxylin. As a positive control, we used human tissue of the tonsil for CD80, CD86 and PD-L1 based on the data sheets. We also used no primary antibody as negative controls in this study, and no specific immunoreactivity was detected in these sections.

To identify immune cells, immunohistochemistry for CD3 (Clone SP7; Thermo Fisher Scientific, Tokyo, Japan), CD20 (clone FB-1; Kindly provided from Dr.

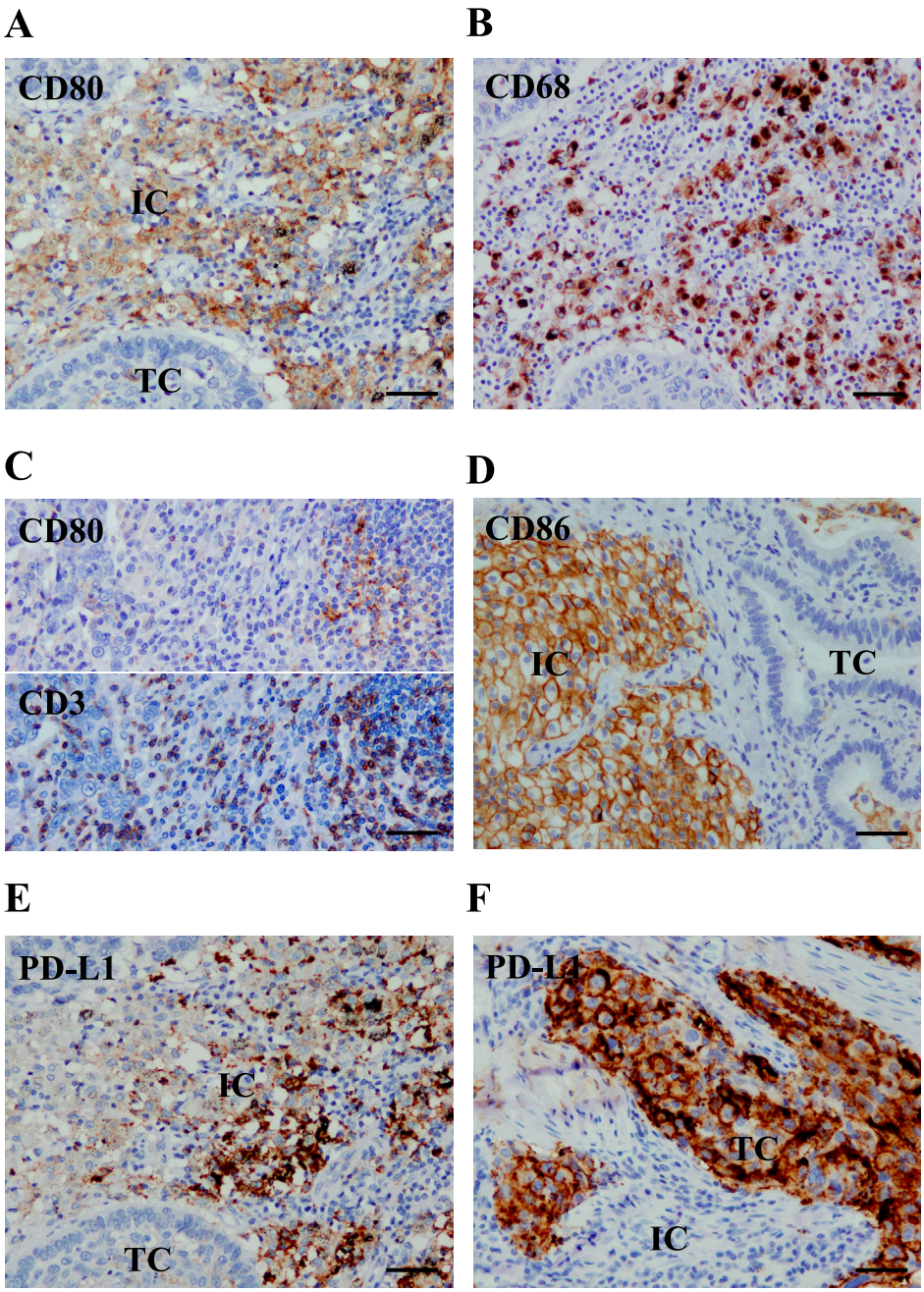

Fig. 1. Immunohistochemistry for CD80, CD86 and PD-L1 in NSCLC. **A**: CD80 was immunolocalized in tumor-infiltrating immune cells (IC) adjacent to tumor cells (TC). **B**: CD68 immunoreactivity in the same area as Fig. 1A. A great majority of CD80-positive cells is CD68-positive macrophages. **C**: Some CD80-positive cells (upper panel) were considered as CD3-positive T lymphocytes (lower panel) in this area. **D**: CD86 immunoreactivity was mainly detected in macrophages in IC. **E**: PD-L1 immunoreactivity was mainly detected in macrophages in IC. Same area as Fig. 1A. **F**: PD-L1 immunoreactivity was detected in TC, but not in IC, in this area. Bar = 50 μm, respectively.

Yuko Hashimoto (Department of Diagnostic Pathology, Fukushima Medical University School of Medicine, Fukushima, Japan)) and CD68 (clone KP1; Agilent, Santa Clara, CA, USA) was also performed with Ventana Benchmark XT platform in this study.

Scoring of immunohistochemistry

CD80 and CD86 immunoreactivity was detected in tumor-infiltrating immune cells in stroma adjacent to the carcinoma cells (IC), and the case that had more than 1% positive stromal cells was considered high [6]. PD-L1 was immunolocalized in tumor cells (TC) and IC, and the case that had more than 1% positive cells in each area was

Table 2. *Association between CD80 and clinicopathological parameters in 75 lung carcinomas*

	CD80 status			CD80 LI	
	high (n = 42)	low (n = 33)	P value	mean ± SEM	P value
Age (years)					
>70	22	19		8.780 ± 1.645	
≤70	20	14	0.654	11.471 ± 2.031	0.301
Gender					
Male	28	15		11.628 ± 1.594	
Female	14	18	0.065	7.812 ± 2.093	0.144
Smoking history					
Smoking	33	19		11.923 ± 1.578	
Non-smoking	9	14	0.050	5.652 ± 1.971	**0.023**
Stage					
0–I	17	23		7.000 ± 1.485	
II–IV	25	10	**0.012**	13.429 ± 2.047	**0.012**
Pathological T factor (pT)					
pTis-1	15	22		6.757 ± 1.553	
pT2-4	27	11	**0.008**	13.158 ± 1.927	**0.119**
Lymph node metastasis					
Positive	11	4		13.333 ± 3.187	
Negative	31	29	0.131	9.167 ± 1.392	0.198
Distant metastasis					
Positive	8	1		14.444 ± 2.940	
Negative	34	32	**0.034**	9.394 ± 1.397	0.205
Histological type					
Adenocarcinoma	23	28		7.059 ± 1.322	
Squamous cell carcinoma	16	4		16.500 ± 2.542	
Others*	3	1	**0.021**	15.000 ± 8.660	**0.003**
Mononuclear infiltration					
high	19	8		13.333 ± 2.201	
low	23	25	0.060	8.125 ± 1.537	0.052
CD86 status					
high	25	15		10.750 ± 1.732	
low	17	18	0.225	9.143 ± 1.939	0.537
PD-L1 (IC) status					
high	33	8		15.122 ± 1.785	
low	9	25	**<0.0001**	3.824 ± 1.195	**<0.0001**
PD-L1 (TC) status					
high	10	1		20.909 ± 4.146	
low	32	32	**0.012**	8.125 ± 1.197	**0.0003**

P-value < 0.05 was significant (in bold).
*; Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

considered high for PD-L1 (TC) and PD-LI (IC), respectively [6, 20, 26]. Immunoreactivity for CD80, CD86, PD-L1 (TC) and PD-L1 (IC) was further semi-quantitatively evaluated by modified labeling index (LI) system according to a previous report [10]. Briefly, the percentage of immunoreactivity (LI) was categorized as 0 (no expression), 10 (up to 10%), 20 (11–20%) until 100 (91–100%) in this study.

Statical analysis

Association between immunohistochemical status of CD80, CD86, PD-L1 (TC) and PD-L1 (IC) and clinicopathological factors were evaluated using Student's t test or a cross-table using the χ^2-test. Over survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional hazard model (Cox). Significant ($P < 0.05$) and borderline-significant ($0.05 \leq P < 0.10$) values were

Table 3. *Association between CD86 and clinicopathological parameters in 75 lung carcinomas*

	CD86 status			CD86 LI	
	high (n = 40)	low (n = 35)	P value	mean ± SEM	P value
Age (years)					
>70	23	18		9.512 ± 1.911	
≤70	17	17	0.598	7.941 ± 1.677	0.547
Gender					
Male	22	21		10.233 ± 2.010	
Female	18	14	0.662	6.875 ± 1.304	0.199
Smoking history					
Smoking	27	25		10.192 ± 1.769	
Non-smoking	13	10	0.713	5.652 ± 1.057	0.104
Stage					
0–I	23	17		10.250 ± 1.977	
II–IV	17	18	0.439	7.143 ± 1.565	0.231
Pathological T factor (pT)					
pTis-1	19	18		7.297 ± 1.533	
pT2-4	21	17	0.734	10.263 ± 2.048	0.252
Lymph node metastasis					
Positive	8	7		6.667 ± 2.108	
Negative	32	28	>0.999	9.333 ± 1.519	0.411
Distant metastasis					
Positive	5	4		5.556 ± 1.757	
Negative	35	31	0.887	9.242 ± 1.437	0.355
Histological type					
Adenocarcinoma	29	22		8.431 ± 1.465	
Squamous cell carcinoma	10	10		10.500 ± 2.945	
Others*	1	3	0.442	5.000 ± 5.000	0.617
Mononuclear infiltration					
high	13	14		7.778 ± 1.949	
low	27	21	0.450	9.375 ± 1.695	0.555
PD-L1 (IC) status					
high	21	20		8.293 ± 1.743	
low	19	15	0.687	9.412 ± 1.932	0.668
PD-L1 (TC) status					
high	6	5		9.091 ± 3.426	
low	34	30	>0.999	8.750 ± 1.400	0.926

*; Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

examined in the multivariate analyses in this study [30]. The statistical analyses were performed using the JMP Pro 15 software (SAS, Institute, Inc, Japan) in this study.

Bioinfomatic analysis

In order to confirm prognostic values of CD80, CD86 and PD-L1 immunoreactivity in the lung cancer patients, we used Kaplan-Meir Plotter for lung cancer which is a large online database containing microarray gene expression data and prognosis of the patients (https://kmplot.com/analysis/index.php?p=service&cancer=lung). Briefly, we selected CD80, CD86 and PD-L1 genes from the database and correlated these expressions with overall survival of lung cancer patients (n = 1,925) by Kaplan-Meier plot.

III. Results

CD80, CD86 and PD-L1 immunolocalization in lung carcinoma tissues

As shown in Fig. 1A, CD80 was immunolocalized in the cytoplasm and membrane of IC in NSCLC. A great majority of the CD80-positive cells was morphologically identified macrophages and CD68 immunoreactivity was also positive (Fig. 1B). In addition, some CD80-positive cells were recognized as CD3-positve T lymphocytes (Fig. 1C) and CD20-positive B lymphocytes. On the other hand, CD80 immunoreactivity was negative in TC (Fig. 1A), non-neoplastic epithelium, and stroma far from TC.

Immunoreactivity of CD86 was also detected in the cytoplasm and membrane of IC (Fig. 1D). The CD86-

Table 4. *Association between PD-L1 (IC) and clinicopathological parameters in 75 lung carcinomas*

	PD-L1 (IC) status			PD-L1 (IC) LI	
	high (n = 41)	low (n = 34)	P value	mean ± SEM	P value
Age (years)					
>70	23	18		8.293 ± 1.518	
≤70	18	16	0.785	8.824 ± 1.829	0.822
Gender					
Male	28	15		10.698 ± 1.677	
Female	13	19	**0.035**	5.625 ± 1.415	**0.030**
Smoking history					
Smoking	32	20		10.000 ± 1.479	
Non-smoking	9	14	0.072	5.217 ± 1.648	0.058
Stage					
0–I	15	25		4.750 ± 1.132	
II–IV	26	9	**0.001**	12.857 ± 1.904	**0.0003**
Pathological T factor (pT)					
pTis-1	14	23		4.054 ± 0.905	
pT2-4	27	11	**0.004**	12.895 ± 1.882	**<0.0001**
Lymph node metastasis					
Positive	13	2		15.333 ± 2.557	
Negative	28	32	**0.005**	6.833 ± 1.223	**0.003**
Distant metastasis					
Positive	7	2		11.111 ± 3.093	
Negative	34	32	0.138	8.182 ± 1.257	0.418
Histological type					
Adenocarcinoma	22	29		5.686 ± 1.094	
Squamous cell carcinoma	15	5		14.500 ± 2.854	
Others*	4	0	**0.009**	15.000 ± 2.887	**0.001**
Mononuclear infiltration					
high	18	9		11.111 ± 2.222	
low	23	25	0.117	7.083 ± 1.296	0.097
PD-L1 (TC) status					
high	11	0		24.545 ± 2.817	
low	30	34	**0.001**	5.781 ± 0.913	**<0.0001**

P-value < 0.05 was significant (in bold).
*; Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

positive cells were mainly macrophages, but some T and B lymphocytes were also positive for CD86. CD86 immunoreactivity was negligible in TC, non-neoplastic epithelium and stroma far from TC. Immunoreactivity of PD-L1 was detected in the cytoplasm and membrane of IC (Fig. 1E) and TC (Fig. 1F).

As shown in Table 2, immunohistochemical CD80 status was high in 42 out of 75 NSCLC (56%) and it was positively associated with stage (P = 0.012), pathological T factor (pT) (P = 0.008), distant metastasis (P = 0.034), histological type (P = 0.021), PD-L1 (IC) status (P < 0.0001) and PD-L1 (TC) status (P = 0.012). Similar tendencies were detected when CD80 immunoreactivity was evaluated as a continuous variable (CD80 LI).

CD86 status was high in 40 out of 75 NSCLC (53%), but it was not significantly associated with any clinicopathological parameters examined (Table 3). As shown in Table 4, PD-L1 (IC) status was high in 41 out of 75 NSCLC (55%), and it was significantly associated with gender (P = 0.035), stage (P = 0.001), pT (P = 0.004), lymph node metastasis (P = 0.005), histological grade (P = 0.009) and PD-L1 (TC) (P = 0.001). Similar tendencies were detected between in PD-L1 (IC) LI and clinicopathological factors. While, PD-L1 (TC) status was high in 11 out of 75 NSCLC (15%), and it was significantly correlated with stage (P = 0.002), pT (P = 0.004) and histological type (P = 0.006) (Table 5).

Association between CD80, CD86 and PD-L1 status and clinical outcome of lung cancer patients

As demonstrated in Fig. 2A, CD80 status was significantly associated with adverse clinical outcome of the patients (P = 0.015 using the log-rank test). No significant relationship was detected between CD80 status and effec-

Table 5. *Association betweenPD-L1 (TC) and clinicopathological parameters in 75 lung carcinomas*

	PD-L1 (TC) status			PD-L1 (TC) LI	
	high (n = 11)	low (n = 64)	P value	mean ± SEM	P value
Age (years)					
>70	4	37		1.463 ± 0.746	
≤70	7	27	0.187	3.235 ± 1.247	0.209
Gender					
Male	8	35		2.558 ± 0.886	
Female	3	29	0.264	1.875 ± 1.139	0.632
Smoking history					
Smoking	9	43		2.692 ± 0.916	
Non-smoking	2	21	0.331	1.304 ± 0.954	0.364
Stage					
0–I	1	39		0.250 ± 0.250	
II–IV	10	25	**0.002**	4.571 ± 1.381	**0.002**
Pathological T factor (pT)					
pTis-1	1	36		0.270 ± 0.270	
pT2-4	10	28	**0.004**	4.211 ± 1.286	**0.004**
Lymph node metastasis					
Positive	4	11		5.333 ± 2.557	
Negative	7	53	0.142	1.500 ± 0.574	**0.027**
Distant metastasis					
Positive	2	7		4.444 ± 2.940	
Negative	9	57	0.495	1.970 ± 0.690	0.253
Histological type					
Adenocarcinoma	3	48		1.373 ± 0.793	
Squamous cell carcinoma	7	13		4.500 ± 1.535	
Others	1	3	**0.006**	2.500 ± 2.500	0.147
Mononuclear infiltration					
high	5	22		2.593 ± 1.144	
low	6	42	0.479	2.083 ± 0.891	0.729

P-value < 0.05 was significant (in bold).
*; Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

tiveness of adjuvant chemotherapies in this study. On the other hand, no significant association was detected between CD86 status and overall survival in these patients (Fig. 2B). PD-L1 (IC) status was significantly associated with worse prognosis of the lung cancer patients (P = 0.046; Fig. 2C), while PD-L1 (TC) was not significantly (P = 0.178) associated with the overall survival in this study (Fig. 2D). When we further examined association between combined CD80/PD-L1 (IC) status and clinical outcome of the patients, high/high group was not significantly associated with worse prognosis compared to low/high (P = 0.586) or high/low (P = 0.845) group (Fig. 2E).

When we analyzed association between CD80, CD86 and PD-L1 mRNA expression and overall survival of lung cancer patients using Kaplan-Meir Plotter for lung cancer, CD80 (P = 0.0054) and PD-L1 (P = 0.023) mRNA expressions were significantly associated with the worse prognosis, but not CD86 (P = 0.39), which is consistent with our immunohistochemical results (Fig. 3).

As shown in Table 6, results of univariate analysis of overall survival using Cox showed distant metastasis (P = 0.002), lymph node metastasis (P = 0.008), stage (P = 0.019), pT (P = 0.028) and CD80 (P = 0.043) status were significant prognostic factors, and PD-L1 (IC) (P = 0.067), and mononuclear infiltration (P = 0.070) were borderline significant. Following multivariate analysis demonstrated that mononuclear infiltration (P = 0.008), CD80 status (P = 0.041) and stage (P = 0.048) were turned out independent prognostic factors for overall survival of NSCLC.

CD80 LI (P = 0.006) was a significant prognostic factor and PD-L1 (IC) (P = 0.062) was borderline significant by Cox. When we used these continuous variables instead of CD80 and PD-L1 (IC) statuses in the multivariate analysis as well as distant metastasis, lymph node metastasis, stage, pT and mononuclear infiltration, only CD80 LI (P = 0.024) and mononuclear infiltration (P = 0.026) were independent prognostic factors in 75 NSCLC patients.

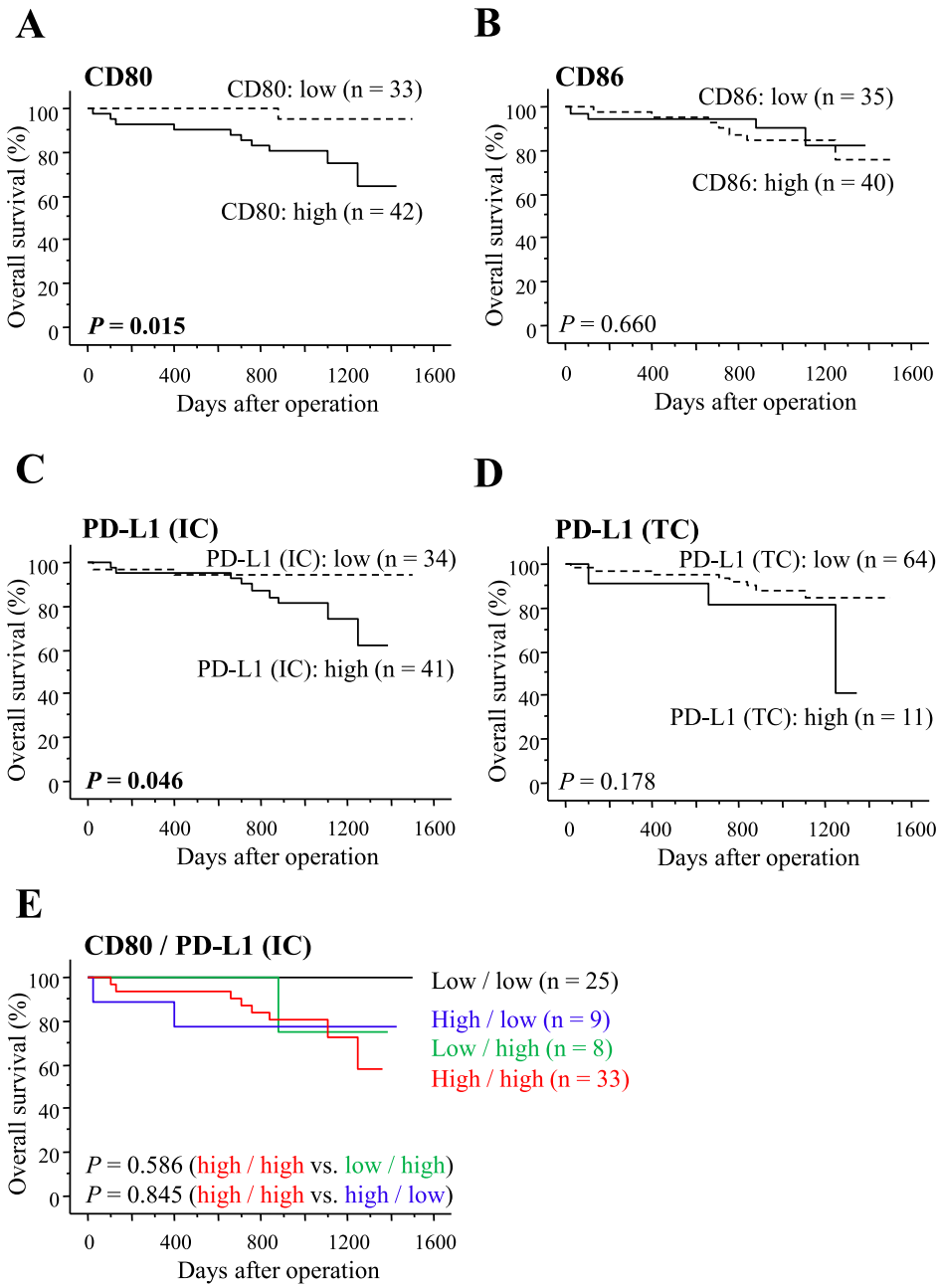

Fig. 2. Overall survival of 75 NSCLC patients according to CD80, CD86, PD-L1 (IC), PD-L1 (TC) and combined CD80/PD-L1 (IC) status. The solid line shows their high group, and the dashed line shows their low group in Fig. 2A–D. *P*-values < 0.05 were considered significant and shown in bold.

IV. Discussion

This is the first study that immunolocalized CD80 and CD86 in lung carcinoma tissues. In this study, CD80 and CD86 immunoreactivities were mainly detected in tumor-infiltrating macrophages in 56% and 53% of NSCLC, respectively. CD80/86 molecules on the surface of antigen presenting cells bind to CD28 on the surface of T lymphocytes, which leads to the activation and differentiation of lymphocytes. However, CTLA-4 competes with CD28 for binding to ligands on the antigen presenting cells with a higher affinity and thereby displaces CD28 from association with CD80/86 [29]. The binding of CTLA-4 to CD80/86 leads to the inhibitory reaction-suppression of the immune response by blocking the T-lymphocyte reducing proliferation of T lymphocytes, inhibiting the activity of Treg lymphocytes, and reducing cytokine secretion and consequently, to immunosuppression [19, 28, 31]. In addition, CD80 specially interacted with PD-L1 and inhibited T cell activation [1, 2]. Therefore, it is suggested that aberrant expression of CD80 and CD86 are involved in the immune microenvironment in NSCLC tissues.

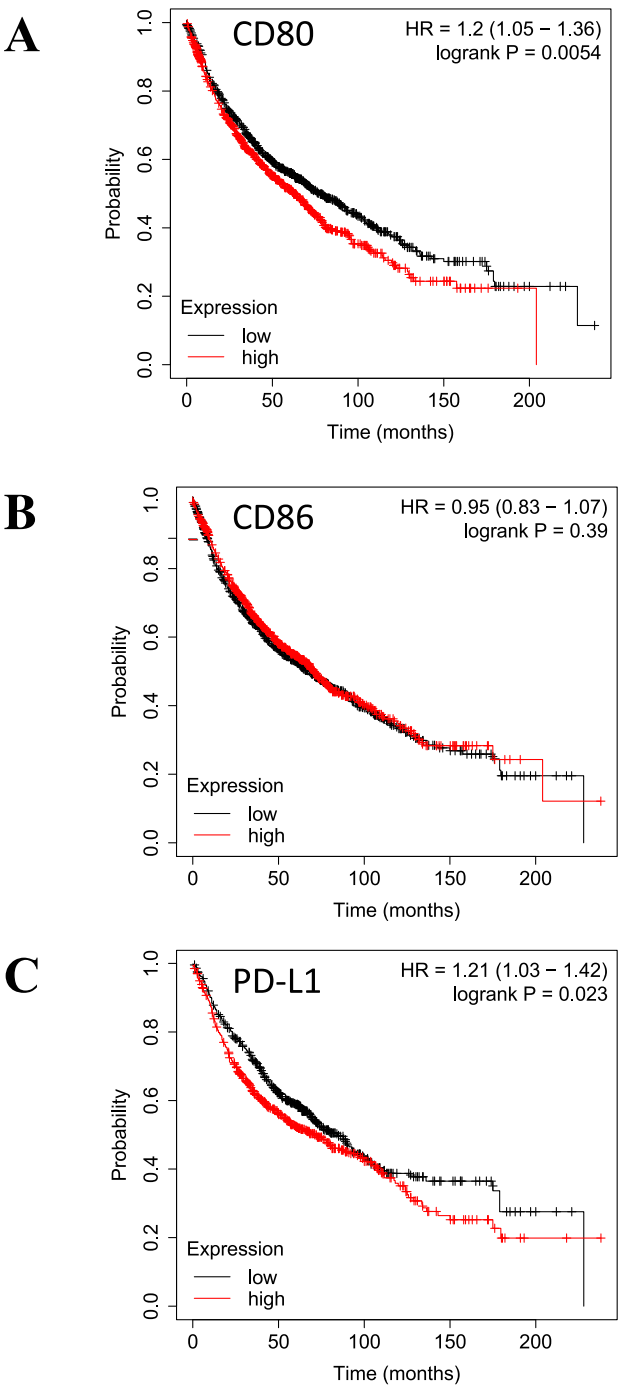

Fig. 3. Association between mRNA expression of CD80, CD86 and PD-L1 and overall survival in lung cancer patients using Kaplan-Meir Plotter for lung cancer. The mRNA expression level in each case was classified into two groups (high (red line) and low (black line)) by the median value (n = 1,925).

In this study, immunohistochemical CD80 status was significantly associated with stage, pT and distant metastasis in NSCLC. Moreover, CD80 status was significantly associated with the worse prognosis, and it turned out an independent prognostic factor. Limited information is available about clinicopathological significance of CD80 in human carcinomas. Previously, Koyama *et al.* [14] reported that almost all patients with gastric carcinoma showed high levels of expression of CD80 and CD86 but the CD80+/CD86+ phenotype was abrogated during tumor invasion and tumor finally acquired the CD80/CD86+ phenotype. In addition, Feng *et al.* [7] reported that CD80 immunoreactivity was a favorable prognostic factor in the gastric adenocarcinoma patients. On the other hand, Wang *et al.* [27] demonstrated that expression level of several immunosuppressive checkpoint molecules, including CD80 and PD-L1, were associated with poor prognosis in pancreatic adenocarcinoma. Considering that clinicopathological significance of CD86 was not evident in the lung carcinoma in this study, it is suggested that CD80 plays an important role to escape from immune attack possibly through CTLA-4 and/or PD-L1 signaling in the lung carcinoma.

Our present study also revealed that CD80-high/PD-L1-high group was not significantly associated with worse prognosis compared to CD80-high/PD-L1-low or CD80-low/PD-L1-high group (Fig. 2E) and PD-L1 (IC) was not an independent prognostic factor. Therefore, it is possible to speculate that CD80 and PD-L1 (IC) signaling pathways are not necessarily independent in NSCLC.

CD80 status was also significantly associated with PD-L1 (IC) status, PD-L1 (TC) status and mononuclear infiltration in this study. In addition, CD80 immunoreactivity was frequently detected in squamous cell carcinoma, and it was marginally associated with smoking history ($P = 0.050$). Previously, Calles *et al.* [4] reported that PD-L1 expression was more frequently detected in squamous cell carcinoma than adenocarcinoma and associated with smoking status, which is generally consistent with our present results of PD-L1. CD80 and PD-L1 were upregulated on antigen presenting cells upon activation [12], and these interacted [1, 2]. PD-L1 was induced by common γ-chain cytokines [13], and INF-γ induced both CD80 and PD-L1 expression [15]. Recently, Cai *et al.* [3] demonstrated that various immune checkpoints, including PD-1, PD-L1, CTLA-4, CD80 and CD86, were significantly higher in the glioma-associated stromal cells, and which was correlated with high-grade gliomas. Therefore, it is suggested both PD-1/PD-L1 and CD80/CTLA-4 pathways are important to regulate immune microenvironment in NSCLC.

Previous studies have shown that PD-L1 expressions in TC and IC are associated with aggressive malignant potential and worse prognosis of NSCLC [21, 23]. In this study, both PD-L1 (TC) and PD-L1 (IC) status was significantly associated with worse prognosis of lung cancer patients, which is in good agreement with these previous reports. Immunohistochemical evaluation of PD-L1 (TC) status is currently used to determine the treatment of PD-1/PD-L1 inhibitors, which is approved by the US Food and Drug Administration [11]. In addition, combined treatment with anti-PD-1/PD-L1 and anti-CATLA-4 is also investigating in several malignant tumors [29], and for instance,

Table 6. *Univariate and multivariate analyses of overall survival in 75 lung cancer patients*

Variable	Univariate	Multivariate	
	P value	*P* value	Relative risk (95% CI)
Distant metastasis (positive/negative)	**0.002**†	0.566	1.683 (0.285–9.934)
Lymph node metastasis (positive/negative)	**0.008**†	0.572	1.733 (0.257–11.680)
Stage (II–IV/0–I)	**0.019**†	**0.048**	11.049 (1.018–119.890)
Pathological T factor (pT) (pT2-4/pTis-1)	**0.028**†	0.448	2.398 (0.250–23.006)
CD80 status (high/low)	**0.043**†	**0.041**	24.306 (1.134–520.841)
PD-L1 (IC) status (high/low)	*0.067*†	0.817	1.307 (0.136–12.584)
Mononuclear infiltration (high/low)	*0.070*†	**0.008**	0.037 (0.003–0.419)
Gender (Male/Female)	0.145		
Histological type* (adenocarcinoma/squamous cell carcinoma)	0.163		
PD-L1 (TC) (high/low)	0.193		
Smoking history (smoking/non-smoking)	0.458		
Patient age (>70/≤70)	0.617		
CD86 status (high/low)	0.661		

Statistical analysis was evaluated by a proportional hazard model (Cox).
P-value < 0.05 and 0.05 ≤ *P*-value < 0.10 were considered significant and borderline significant, and were listed in bold and italic, respectively.
†; Significant ($P < 0.05$) and borderline-significant ($0.05 \leq P < 0.10$) values were examined in the multivariate analyses in this study.
95% CI, 95% confidence interval.
*; Other histological types (n = 4) rather than adenocarcinoma and squamous cell carcinoma were excluded in this analysis.

combination with nivolumab (PD-1 inhibitor), ipilimumab (CTLA-4 inhibitor) and chemotherapy seems to be superior first-line immunotherapy for patients with advanced non-small cell lung carcinoma [16]. PD-L1 inhibitor durvalumab blocks PD-L1 binding to CD80 as well as PD-1, and clinical trial to investigate effects of durvalumab with or without tremelimumab (CTLA-4 inhibitor) versus standard chemotherapy is also undergoing in non-small cell lung cancer [8]. Appropriate biomarker for the treatment of anti-CTLA-4 inhibitors is currently unknown, and further examinations are required to clarify the biological functions of CD80 to improve the immunotherapy in NSCLC patients.

In summary, we immunolocalized CD80 and CD86 in 75 NSCLC tissues. CD80 status was high in 56% of lung carcinomas and it was positively associated with stage, pT, distant metastasis, histological type, intratumoral mononuclear infiltration, PD-L1 (IC) status and PD-L1 (TC) status. Moreover, CD80 status was significantly associated with poor prognosis of the patients, and multivariate analysis turned out it as an independent prognostic factor. CD86 status was high in 53% of the cases, but it was not significantly associated with any clinicopathological parameters. These findings suggest that CD80 is a potent worse prognostic factor possibly in association with escape from immune attack in NSCLC.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. References

1. Butte, M. J., Keir, M. E., Phamduy, T. B., Sharpe, A. H. and Freeman, G. J. (2007) Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27; 111–122.
2. Butte, M. J., Peña-Cruz, V., Kim, M. J., Freeman, G. J. and Sharpe, A. H. (2008) Interaction of human PD-L1 and B7-1. *Mol. Immunol.* 45; 3567–3572.
3. Cai, X., Yuan, F., Zhu, J., Yang, J., Tang, C., Cong, Z., *et al.* (2021) Glioma-Associated Stromal Cells Stimulate Glioma Malignancy by Regulating the Tumor Immune Microenvironment. *Front. Oncol.* 11; 672928.
4. Calles, A., Liao, X., Sholl, L. M., Rodig, S. J., Freeman, G. J., Butaney, M., *et al.* (2015) Expression of PD-1 and Its Ligands, PD-L1 and PD-L2, in Smokers and Never Smokers with KRAS-Mutant Lung Cancer. *J. Thorac. Oncol.* 10; 1726–1735.
5. Chen, L., Cao, M. F., Zhang, X., Dang, W. Q., Xiao, J. F., Liu, Q., *et al.* (2019) The landscape of immune microenvironment in lung adenocarcinoma and squamous cell carcinoma based on PD-L1 expression and tumor-infiltrating lymphocytes. *Cancer Med.* 8; 7207–7218.
6. Fehrenbacher, L., Spira, A., Ballinger, M., Kowanetz, M., Vansteenkiste, J., Mazieres, J., *et al.* (2016) Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet* 387; 1837–1846.
7. Feng, X. Y., Lu, L., Wang, K. F., Zhu, B. Y., Wen, X. Z., Peng, R. Q., *et al.* (2019) Low expression of CD80 predicts for poor prognosis in patients with gastric adenocarcinoma. *Future Oncol.* 15; 473–483.
8. Garon, E. B., Cho, B. C., Reinmuth, N., Lee, K. H., Luft, A., Ahn, M. J., *et al.* (2021) Patient-Reported Outcomes with Durvalumab With or Without Tremelimumab Versus Standard Chemotherapy as First-Line Treatment of Metastatic Non-Small-Cell Lung Cancer (MYSTIC). *Clin. Lung Cancer* 22; 301–312.

9. Greaves, P. and Gribben, J. G. (2013) The role of B7 family molecules in hematologic malignancy. *Blood* 121; 734–744.
10. Hayashi, C., Takagi, K., Sato, A., Yamaguchi, M., Minemura, H., Miki, Y., *et al.* (2021) D-2-hydroxyglutarate dehydrogenase in breast carcinoma as a potent prognostic marker associated with proliferation. *Histol. Histopathol.* Online ahead of print.
11. Herbst, R. S., Baas, P., Kim, D. W., Felip, E., Pérez-Gracia, J. L., Han, J. Y., *et al.* (2016) Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* 387; 1540–1550.
12. Keir, M. E., Butte, M. J., Freeman, G. J. and Sharpe, A. H. (2008) PD-1 and its ligands in tolerance and immunity. *Annu. Rev. Immunol.* 26; 677–704.
13. Kinter, A. L., Godbout, E. J., McNally, J. P., Sereti, I., Roby, G. A., O'Shea, M. A., *et al.* (2008) The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J. Immunol.* 181; 6738–6746.
14. Koyama, S., Maruyama, T., Adachi, S. and Nozue, M. (1998) Expression of costimulatory molecules, B7-1 and B7-2 on human gastric carcinoma. *J. Cancer Res. Clin. Oncol.* 124; 383–388.
15. Li, J., Yang, Y., Inoue, H., Mori, M. and Akiyoshi, T. (1996) The expression of costimulatory molecules CD80 and CD86 in human carcinoma cell lines: its regulation by interferon gamma and interleukin-10. *Cancer Immunol. Immunother.* 43; 213–219.
16. Liu, L., Bai, H., Wang, C., Seery, S., Wang, Z., Duan, J., *et al.* (2021) Efficacy and Safety of First-Line Immunotherapy Combinations for Advanced NSCLC: A Systematic Review and Network Meta-Analysis. *J. Thorac. Oncol.* 16; 1099–1117.
17. Okazaki, T., Chikuma, S., Iwai, Y., Fagarasan, S. and Honjo, T. (2013) A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat. Immunol.* 14; 1212–1218.
18. Pauken, K. E., Torchia, J. A., Chaudhri, A., Sharpe, A. H. and Freeman, G. J. (2021) Emerging concepts in PD-1 checkpoint biology. *Semin. Immunol.* 15; 101480.
19. Rowshanravan, B., Halliday, N. and Sansom, D. M. (2018) CTLA-4: a moving target in immunotherapy. *Blood* 131; 58–67.
20. Scheel, A. H., Ansén, S., Schultheis, A. M., Scheffler, M., Fischer, R. N., Michels, S., *et al.* (2016) PD-L1 expression in non-small cell lung cancer: Correlations with genetic alterations. *Oncoimmunology* 5; e1131379.
21. Sepesi, B., Cuentas, E. P., Canales, J. R., Behrens, C., Correa, A. M., Vaporciyan, A., *et al.* (2017) Programmed Death Cell Ligand 1 (PD-L1) Is Associated With Survival in Stage I Non-Small Cell Lung Cancer. *Semin. Thorac. Cardiovasc. Surg.* 29; 408–415.
22. Shtivelman, E., Hensing, T., Simon, G. R., Dennis, P. A., Otterson, G. A., Bueno, R., *et al.* (2014) Molecular pathways and therapeutic targets in lung cancer. *Oncotarget* 5; 1392–1433.
23. Sumitomo, R., Hirai, T., Fujita, M., Murakami, H., Otake, Y. and Huang, C. L. (2019) PD-L1 expression on tumor-infiltrating immune cells is highly associated with M2 TAM and aggressive malignant potential in patients with resected non-small cell lung cancer. *Lung Cancer* 136; 136–144.
24. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., *et al.* (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* 71; 209–249.
25. Suzuki, T., Inoue, S., Kawabata, W., Akahira, J., Moriya, T., Tsuchiya F., *et al.* (2001) EBAG9/RCAS1 in human breast carcinoma: a possible factor in endocrine-immune interactions. *Br. J. Cancer* 85; 1731–1737.
26. Takada, K., Toyokawa, G., Okamoto, T., Shimokawa, M., Kozuma, Y., Matsubara, T., *et al.* (2017) A Comprehensive Analysis of Programmed Cell Death Ligand-1 Expression With the Clone SP142 Antibody in Non-Small-Cell Lung Cancer Patients. *Clin. Lung Cancer* 18; 572–582.
27. Wang, W., Yan, L., Guan, X., Dong, B., Zhao, M., Wu, J., *et al.* (2020) Identification of an Immune-Related Signature for Predicting Prognosis in Patients With Pancreatic Ductal Adenocarcinoma. *Front. Oncol.* 10; 618215.
28. Wei, S. C., Duffy, C. R. and Allison, J. P. (2018) Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discov.* 8; 1069–1086.
29. Wojtukiewicz, M. Z., Rek, M. M., Karpowicz, K., Górska, M., Polityńska, B., Wojtukiewicz, A. M., *et al.* (2021) Inhibitors of immune checkpoints-PD-1, PD-L1, CTLA-4-new opportunities for cancer patients and a new challenge for internists and general practitioners. *Cancer Metastasis Rev.* doi: 10.1007/s10555-021-09976-0. Online ahead of print.
30. Yamaguchi, M., Takagi, K., Sato, A., Miki, Y., Miyashita, M., Sasano, H., *et al.* (2020) Rac1 activation in human breast carcinoma as a prognostic factor associated with therapeutic resistance. *Breast Cancer* 27; 919–928.
31. Zhao, Y., Yang, W., Huang, Y., Cui, R., Li, X. and Li, B. (2018) Evolving Roles for Targeting CTLA-4 in Cancer Immunotherapy. *Cell. Physiol. Biochem.* 47; 721–734.
32. Zimmermann, S., Dziadziuszko, R. and Peters, S. (2014) Indications and limitations of chemotherapy and targeted agents in non-small cell lung cancer brain metastases. *Cancer Treat. Rev.* 40; 716–722.