



CrossMark

Tumour-infiltrating lymphocytes are correlated with higher expression levels of PD-1 and PD-L1 in early breast cancer

Atsuko Kitano,¹ Makiko Ono,^{1,2} Masayuki Yoshida,³ Emi Noguchi,¹ Akihiko Shimomura,¹ Tatsunori Shimoi,¹ Makoto Kodaira,¹ Mayu Yunokawa,¹ Kan Yonemori,¹ Chikako Shimizu,¹ Takayuki Kinoshita,⁴ Yasuhiro Fujiwara,¹ Hitoshi Tsuda,⁵ Kenji Tamura¹

To cite: Kitano A, Ono M, Yoshida M, *et al.* Tumour-infiltrating lymphocytes are correlated with higher expression levels of PD-1 and PD-L1 in early breast cancer. *ESMO Open* 2017;2:e000150. doi:10.1136/esmooopen-2016-000150

Received 22 December 2016
Revised 10 January 2017
Accepted 10 January 2017

¹Department of Breast and Medical Oncology, National Cancer Center Hospital, Tokyo, Japan

²Department of Medical Oncology, Cancer Institute Hospital, Tokyo, Japan

³Division of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan

⁴Department of Breast Surgery, National Cancer Center Hospital, Tokyo, Japan

⁵Department of Basic Pathology, National Defense Medical College, Saitama, Japan

Correspondence to

Dr Kenji Tamura; ketamura@ncc.go.jp

ABSTRACT

Background The presence of tumour-infiltrating lymphocytes (TILs) is a favourable prognostic factor in patients with early breast cancer. Programmed cell death-1 (PD-1) and its ligand PD-L1 are associated with a variety of adverse features. The purpose of this study was to clarify the relationships between TILs, PD-1 and PD-L1 as well as their prognostic implications in early breast cancer.

Methods We investigated 180 patients with breast cancer who received neoadjuvant chemotherapy and underwent subsequent surgery for stage II–III invasive breast carcinoma between 1999 and 2007. TIL expression was classified as low or high using a previously reported scoring model. PD-1 and PD-L1 expression levels were determined by immunohistochemistry. The correlation between PD-1 expression in TILs and PD-L1 expression in cancer cells was also investigated.

Results Higher tumour grade was significantly correlated with PD-L1 expression in tumours ($p<0.0001$). PD-1 and PD-L1 expression levels were associated with tumour subtype and were highest in triple-negative tumours ($p<0.0001$). Furthermore, expression of each of PD-1 and PD-L1 was significantly correlated with higher TIL expression and pathological complete response (pCR) ($p<0.0001$). PD-L1 expression in cancer cells was significantly correlated with PD-1 expression in TILs ($p=0.03$). The correlations between pCR and expression of each of PD-L1 and PD-1 were not significant.

Conclusion Expression of PD-L1 and PD-1 in early breast cancer is associated with higher TIL scores and pCR; conversely, expression of these proteins correlates with poor prognostic clinicopathological factors such as tumour grade and subtype. TILs, PD-1 and PD-L1 can potentially predict the response to treatment.

INTRODUCTION

Blocking the immune checkpoint receptor ‘programmed cell death-1’ (PD-1), and its ligands PD-L1 and PD-L2, is one of the most promising strategies in cancer immunotherapy.^{1–4} PD-L1 and PD-L2 are expressed by antigen-presenting cells such as macrophages or B-cells. After binding to its ligands, PD-1 attenuates lymphocyte activation and promotes

Key questions

What is already known about this subject?

- ▶ The presence of tumour-infiltrating lymphocytes (TILs) is a prognostic factor in patients with early breast cancer.
- ▶ Programmed cell death-1 (PD-1) and its ligand PD-L1 are associated with a variety of adverse features.

What does this study add?

- ▶ Expression of PD-L1 and PD-1 in early breast cancer is associated with higher TIL expression and pathological complete response (pCR).
- ▶ PD-L1 expression in cancer cells was significantly correlated with PD-1 expression in TILs.

How might this impact on clinical practice?

- ▶ As immune-targeting therapies based on PD-1/PD-L1 blockade are being developed, understanding the roles of TILs, PD-1 and PD-L1 in breast cancer is critical. Our data show that PD-L1 and PD-1 are associated with poor clinicopathological features and (paradoxically) are indicators of good prognosis, higher TIL expression and pCR.

T-regulatory cell development and function, which in turn downregulates the immune response.^{2–5}

Tumour-infiltrating lymphocytes (TILs) comprise various types of lymphocytes; their activities are regulated by complex immune system activator and inhibitor pathways.^{5–6} TILs have long been associated with clinical treatment response in various solid tumours.⁷ TIL expression in the breast cancer tumour microenvironment has long been recognised as a favourable prognostic factor,^{8–9} especially in tumours with highly proliferative characteristics such as those that are triple-negative breast cancer (TNBC) or are human epidermal growth factor receptor 2 (HER2) positive.^{10–12} Moreover, higher TIL expression is a predictive marker of pathological complete response

(pCR) following neoadjuvant chemotherapy in TNBC, as previously reported by our group and others.^{13–16}

Recent studies have revealed that PD-1 and PD-L1 expression levels in breast tumours are associated with adverse clinicopathological features.^{17–18} Based on these findings, several clinical trials targeting PD-1 and PD-L1 were recently conducted.¹⁹ Among patients who received neoadjuvant chemotherapy, PD-L1 expression in the epithelium was a significant predictor of pCR.²⁰ Sabatier *et al* also reported that high PD-1 and PD-L1 expression was associated with better overall survival and higher pCR rates in TNBC.²¹

There has been lack of studies aimed at clarifying the relationship between PD-1, PD-L1 and TILs in patients with similar-stage breast cancer. Furthermore, the associations between the expression of these proteins and clinical outcomes in breast cancer subtypes other than TNBC have not been investigated. Therefore, we performed this study to identify clinicopathological factors associated with PD-L1 expression in tumour cells and PD-1 expression in TILs in early breast cancer and to reveal the association between clinical outcomes and PD-L1 or PD-1 expression.

MATERIAL AND METHODS

Patients and tissue samples

Our patient cohort was the same as that employed in our previous study on the association between TILs and neoadjuvant chemotherapy response.¹³ One hundred and eighty patients who received neoadjuvant chemotherapy and subsequent surgery for stage II–III invasive breast cancer between 1999 and 2007 with available surgical core needle biopsy (CNB) specimens were analysed. Clinical characteristics of all patients were obtained from their medical charts. All patients received anthracycline and taxane-based neoadjuvant chemotherapy using sequential or concurrent regimens (adriamycin 60 mg/m² plus cyclophosphamide 600 mg/m² (AC) or cyclophosphamide 600 mg/m² plus epirubicin 100 mg/m²/5-fluorouracil 600 mg/m² (FEC), followed by weekly paclitaxel 80 mg/m² or triweekly docetaxel 75 mg/m², or Adriamycin 50 mg plus docetaxel 60 mg/m²) an anthracycline-based regimen alone (AC or FEC) or a taxane-based regimen (weekly paclitaxel or triweekly docetaxel) alone. Trastuzumab was not used for 42 patients with HER2 because it was not approved for neoadjuvant chemotherapy in Japan at that time. The median follow-up time was 115 months (range: 4–202 months). All specimen were formalin fixed and paraffin embedded, and 4 µm thick sections were prepared for H&E staining. The immunohistochemistry (IHC) samples were reviewed by two observers including an experienced pathologist. The definition of pCR was ypT0/is ypN0/+ according to the National Adjuvant Breast and Bowel Project protocol.

The present study was approved by the Institutional Review Board of the National Cancer Center Hospital.

Histopathological evaluation

Histopathological assessment of predictive factors was performed for the CNB specimens. Histopathological parameters included histological grade,²² histological type,²³ presence of TILs, PD-1 expression in TILs and PD-L1 expression in tumour cells. TILs were evaluated using a previously reported TIL scoring model.¹³ Briefly, the TIL score is the sum of proportional scores (areas infiltrated by lymphocytes) and intensity scores (intensity of lymphatic infiltration) for each tumour. TIL scores of 3–5 were classified as high, whereas scores of 0–2 were classified as low.

IMMUNOHISTOCHEMISTRY

IHC of the CNB specimens was evaluated using the following primary antibodies: antioestrogen receptor (ER) (clone 1D5; Dako), antiprogestosterone receptor (PgR) (clone PgR636; Dako), anti-HER2 (polyclonal; HercepTest II; Dako), antihuman PD-1 monoclonal antibody (clone NAT105, 1:100 dilution; Abcam, Cambridge, UK) and antihuman PD-L1 polyclonal antibody (#4059, 1:200 dilution; ProScience, Poway, California, USA). Antigen retrieval for PD-1 was performed in TRS buffer (Dako) at 121°C for 10 min; no antigen retrieval was performed for PD-L1. The peroxidase-based Envision System (Dako) was used for secondary detection of the antibody; 3, 3'-diaminobenzidine tetrahydrochloride was used for the immunoperoxidase reaction, and the samples were counterstained with haematoxylin. Tonsil tissues were used as positive controls, while similarly treated samples absent the primary antibody were used as secondary controls. ER and PgR were judged as positive if the Allred score was ≥3 and as negative if the score was ≤2.²⁴ HER2 overexpression was judged according to the American Society of Clinical Oncology/College of American Pathologists guidelines of 2007.²⁵ TNBC tumours were, by definition, negative for ER, PgR and HER2. The HR+/HER2– subtype was defined as positive for ER or PgR and negative for HER2, while the HR–/HER2+ subtype was defined as negative for ER and PgR but positive for HER2. PD-1 expression in TILs and PD-L1 expression in tumours were considered positive if any positively stained cells were present (figure 1).

Statistical analysis

Association between clinicopathological characteristics and expression of PD-1 and/or PD-L1 were evaluated using the χ^2 test or Fisher's exact test. Correlations between PD-1 expression in TILs and PD-L1 expression in tumours were evaluated using the χ^2 test. Univariate and multivariate binary logistic regression analyses were performed to identify independent predictive factors of pCR. Survival curves were constructed using the Kaplan-Meier method, and statistical differences between survival curves were calculated by using the log-rank test. Statistical significance was set at $p < 0.05$.

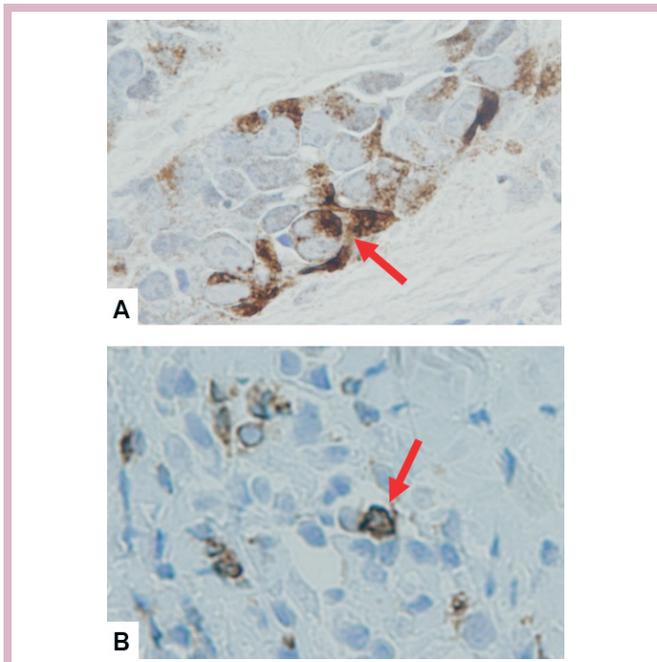


Figure 1 Immunohistochemical features of PD-L1 and PD-1 expression. (A) PD-L1 expression in tumour cells; the arrow indicates PD-L1 staining. (B) PD-1 expression on tumour-infiltrating lymphocytes; the arrow indicates PD-1 staining. PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1.

for all the analyses, which were performed using the JMP 1.2 software, V.12.

RESULTS

Patient characteristics

Table 1 shows the characteristics of the 180 patients from whom evaluable tumours were obtained using CNB. Ninety-two tumours were TNBC, 42 were HR⁻/HER2⁺ and 46 were HR⁺/HER2⁻. Patients with TNBC and HR⁻/HER2⁺ tumour subtypes had significantly higher tumour grades ($p < 0.001$). Ninety-eight tumours (54%) had high TIL expression; high TIL-score tumours were significantly more prevalent among TNBC and HR⁻/HER2⁺ subtypes, as we reported previously.¹³ PD-1 expression was detected in TILs of 40 tumours (22%), whereas PD-L1 expression was detected in 62 tumours (34%). The PD-L1 expression rate was highest in TNBC tumours, followed by HR⁻/HER2⁺ and HR⁺/HER2⁻ subtypes ($p = 0.004$). p53 expression and the apoptosis score were higher in TNBC and HR⁻/HER2⁺ subtypes; the correlation between these factors and the three tumour subtypes was significant ($p < 0.001$ for both factors).¹³

Correlation between clinicopathological factors and PD-L1 expression in tumour cells or PD-1 expression in TILs

The correlations between clinicopathological factors and PD-L1 expression in tumour cells or PD-1 expression in TILs are shown in table 2. PD-L1 expression was evaluated in 166 tumours, while PD-1 expression

in TILs was evaluated in 164 tumours among the 180 screened samples. Higher tumour grade was significantly correlated with PD-L1 expression in tumours ($p < 0.0001$). The tumour subtype also correlated with PD-L1 expression in tumours, particularly TNBC ($p < 0.0001$). PD-L1 expression in tumour cells was detected in all patients who achieved pCR; moreover, there was a significant correlation between achievement of pCR and PD-L1 expression in tumours ($p < 0.0001$). Higher TIL scores and p53 expression were significantly correlated with PD-L1 expression in tumour cells ($p < 0.0001$ and $p = 0.0074$, respectively). Clinicopathological factors that were statistically correlated with PD-L1 expression in tumour cells likewise correlated with PD-1 expression in TILs; these included tumour grade, tumour subtype, pCR, higher TIL score and p53 expression.

Correlation between PD-L1 expression in tumour cells and PD-1 expression in TILs

One hundred and sixty-two tumours were evaluable for both PD-L1 expression in tumour cells and PD-1 expression in TILs among the 180 screened samples (table 3). PD-L1 expression in tumour cells was significantly associated with PD-1 expression in TILs ($p = 0.03$). Among PD-L1-positive tumours, 21 (34%) also expressed PD-1. The rate of co-expression of PD-L1 in tumour cells and PD-1 expression in TILs from the same specimen was 13% (21/162).

Correlation between PD-L1, PD-1 expression and TILs with pCR by tumour subtype

The correlations between pCR and each of PD-L1 expression, PD-1 expression and TIL score were evaluated by using the χ^2 test (table 4). PD-L1 expression in tumour cells and pCR were marginally correlated in HR⁻/HER2⁺ tumours, although the relationship was not significant ($p = 0.08$). No correlations between PD-1 expression in TILs and pCR were evident in any of the tumour subtypes. High TIL scores and pCR were significantly correlated in TNBCs, which is consistent with our previous study¹³ ($p = 0.05$); however, no such significant differences were observed in the HR⁻/HER2⁺ or HR⁺/HER2⁻ subtypes.

Survival outcome according to PD-L1 expression in tumour cells and PD-1 expression in TILs

No significant differences in the rates of disease-free and overall survival were identified according to PD-L1 or PD-1 expression during the follow-up period (median: 115 months; range: 4–202 months) (figure 2). Furthermore, there were no statistically significant differences in survival rates according to PD-L1 and PD-1 expression by tumour subtype (data not shown).

DISCUSSION

Our data clarified the relationship between expression of PD-L1 in tumour cells and PD-1 in TILs. The association between certain clinicopathological factors and PD-L1 expression in breast cancer has been reported in

Table 1 Patient characteristics

		All (n=180)	TNBC (n=92)	HR-/HER2+ (n=42)	HR+/HER2- (n=46)	p
Age: median (range)		54 (23–76)	52 (23–76)	55 (31–71)	55 (31–71)	0.36
Patients, n (%)						
T	1	2 (1)	2 (2)	0 (0)	0 (0)	0.37
	2	91 (51)	48 (53)	17 (41)	26 (56)	
	3	54 (30)	27 (29)	16 (38)	11 (24)	
	4	33 (18)	15 (16)	9 (21)	9 (20)	
N	0	93 (52)	45 (49)	24 (57)	24 (52)	0.96
	1	67 (37)	35 (38)	14 (33)	18 (39)	
	2	16 (9)	10 (11)	3 (7)	3 (7)	
	3	4 (2)	2 (2)	1 (3)	1 (2)	
Stage	II	109 (61)	56 (61)	25 (60)	28 (61)	0.99
	III	71 (39)	36 (39)	17 (40)	18 (39)	
Grade	1	5 (3)	1 (1)	0 (0)	4 (9)	<0.001
	2	53 (29)	9 (10)	8 (19)	36 (78)	
	3	122 (68)	82 (89)	34 (81)	6 (3)	
TIL	Low	82 (46)	25 (27)	19 (45)	38 (83)	0.002
	High	98 (54)	67 (73)	23 (55)	8 (17)	
PD-1 expression in TIL	Positive	40 (22)	26 (28)	8 (19)	6 (13)	0.07
	Negative	124 (69)	56 (61)	32 (76)	36 (78)	
	Unknown	16 (9)	10 (11)	2 (5)	4 (9)	
PD-L1 expression in tumour cells	Positive	62 (34)	40 (43)	13 (31)	9 (20)	0.004
	Negative	104 (58)	41 (45)	28 (67)	35 (76)	
	Unknown	14 (8)	11 (12)	1 (2)	2 (4)	
pCR	Positive	41 (23)	29 (32)	9 (21)	3 (7)	0.004
	Negative	139 (77)	63 (68)	33 (79)	43 (93)	
p53	Positive	96 (53)	58 (63)	26 (62)	12 (26)	<0.001
	Negative	84 (47)	34 (37)	16 (38)	34 (74)	
Apoptosis	0	59 (33)	22 (24)	8 (19)	29 (63)	<0.001
	1	81 (45)	51 (55)	14 (33)	16 (35)	
	2	40 (22)	19 (21)	20 (48)	1 (2)	

HER2, human epidermal growth factor receptor 2; HR, hormone receptors; pCR, pathological complete response; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; T, tumour; TIL, tumour-infiltrating tumour cells; TNBC, triple-negative breast cancer; N, node.

various studies.^{17–26} A meta-analysis revealed that PD-L1 expression in breast tumours is a poor prognostic clinicopathological factor, as it is related to lymph node metastasis, poor nuclear grade and negative oestrogen receptor status.²⁷ The present study similarly concluded that PD-L1 expression in tumours correlates with both poor grade and more aggressive tumour subtypes. Our findings also suggest that PD-1 expression in TILs is likewise related to poorly prognostic clinicopathological factors. Moreover, expression of both PD-L1 in tumour cells and PD-1 in TILs were related to a high TIL score and, paradoxically, a higher pCR rate.

Although PD-L1 expression in tumours is considered a diagnostic marker for anti PD-1 antibodies (such as pembrolizumab) in non-small cell lung cancer,²⁸ it is still unclear whether PD-L1 expression is a prognostic marker for all immune-targeting therapies based on PD-1/PD-L1 blockade in various types of cancer. To ensure a response to PD-1/PD-L1 checkpoint blockade, a new framework based on the presence or absence

of TILs and PD-L1 expression was required.^{29–30} Four categories of tumours according to TIL and PD-L1 expression were proposed as a measure of predicting their response to immune checkpoint inhibitors: type I (PD-L1+/TIL+; adaptive immune resistance), type II (PD-L1-/TIL-; immunological ignorance), type III (PD-L1+/TIL-; intrinsic induction) and type IV (PD-L1-/TIL+; tolerance).³¹ The relationship between PD-L1 expression and TILs in breast cancer was also reported in some retrospective clinical investigations,^{20–32} although no published data exist regarding the predictive value of PD-L1 expression and TILs for immune checkpoint inhibitor therapy to our knowledge.

Previous studies showed that PD-L1 expression correlated with that of TILs and to neoadjuvant chemotherapy response, particularly in HR- tumours and TNBCs.²⁰ Our data suggest that PD-L1 expression, as well as TIL scores, have a correlative trend with pCR. We also revealed that PD-1 expression in TILs correlated with the TIL score.

Table 2 Correlation between clinicopathological factors and PD-L1 expression in tumour cells or PD-1 expression in TILs

		No. of patients (%)			No. of patients (%)		
		PD-L1 expression (n=166)		p	PD-1 expression (n=164)		p
		Positive	Negative		Positive	Negative	
Age (years)	≤50	25 (43)	33 (57)	0.28	15 (27)	41 (57)	0.61
	>50	37 (35)	70 (65)		25 (24)	83 (65)	
T	1/2	53 (61)	34 (39)	0.13	54 (62)	33 (38)	0.19
	3/4	39 (49)	40 (51)		40 (52)	37 (48)	
N	0	47 (55)	39 (45)	0.84	49 (56)	38 (44)	0.78
	≥1	45 (56)	35 (44)		45 (58)	32 (42)	
Stage	II	40 (40)	61 (60)	0.18	26 (25)	76 (75)	0.21
	III	22 (34)	42 (66)		14 (23)	48 (77)	
Grade	1 or 2	15 (28)	39 (72)	<0.0001	9 (17)	43 (83)	<0.0001
	3	47 (42)	64 (58)		31 (28)	81 (72)	
Subtype	TNBC	40 (49)	41 (51)	<0.0001	26 (32)	56 (51)	<0.0001
	HR-/HER2+	13 (33)	27 (67)		8 (20)	32 (67)	
	HR+/HER2-	9 (21)	35 (79)		6 (14)	36 (79)	
pCR	Yes	33 (100)	0 (0)	<0.0001	34 (100)	0 (0)	<0.0001
	No	59 (44)	74 (56)		60 (46)	70 (54)	
TIL	High	66 (76)	21 (24)	<0.0001	67 (78)	19 (22)	<0.0001
	Low	26 (33)	53 (67)		27 (35)	51 (65)	
p53	Positive	58 (67)	29 (33)	0.0074	59 (70)	25 (30)	0.002
	Negative	34 (43)	45 (57)		35 (44)	45 (56)	
Apoptosis	Score 0, 1	69 (54)	58 (46)	0.61	71 (56)	55 (44)	0.65
	Score 2	23 (59)	16 (41)		23 (61)	15 (39)	

HER2, human epidermal growth factor receptor 2; HR, hormone receptors; pCR, pathological complete response; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; TIL, tumour-infiltrating tumour cells; TNBC, triple-negative breast cancer.

However, we did not reveal PD-L1 expression in tumour cells, PD-1 expression in TILs and TIL scores to be predictive markers of pCR except for TIL scores in patients with TNBC, which our group also reported previously.¹³ PD-L1 expression in tumours marginally correlated with pCR in HR-/HER2+ tumour, although not significantly.

PD-L1 and PD-1 were shown to be expressed concurrently in various tumours, including endometrial tumours (79%), malignant melanomas (58%), bladder cancer (55%), non-small cell lung carcinomas (43%), ovarian cancer (36%) and kidney cancer (33%).³³ It was also reported that the rate of concurrent PD-L1 and PD-1 expression in breast cancers regardless of tumour subtype was 29%; however, the rate in TNBCs was significantly higher than that in

non-TNBCs (45% vs 13%–17%, respectively; $p=0.001$).³³ In our study, the rate of concurrent PD-L1 and PD-1 expression was 34%, which was similar to rates previously reported. Moreover, we found that PD-L1 expression in tumours was significantly associated with PD-1 expression in TILs. A previous study showed that PD-1 expression in TILs was associated with a higher rate of mutations, whereas PD-L1 expression in tumours showed the opposite association.³³ Breast cancers with mutated *TP53* exhibit higher PD-1 in TILs compared with breast cancers that harbour other mutations (eg, *PIK3CA*) or those without mutations.³³ In contrast, PD-L1 expression in tumour cells does not correlate with mutation status.³³

TIL presence has been reported to be a positive prognostic factor for TNBC and HER2-positive breast cancer^{8 10 22}; however, few studies have explored the role of PD-L1 and PD-1 expression in the survival of patients with breast cancer. Sabatier *et al* reported that PD-L1 mRNA upregulation was associated with better survival and response to chemotherapy in patients with TNBC.²¹ Schalper *et al* also reported that PD-L1 mRNA expression is associated with increased TILs and improved recurrence-free survival rates.³² Our data showed no survival differences according to PD-L1 or PD-1 expression in any of the breast cancer subtypes. One reason for this discrepancy may be owing to the use of IHC to evaluate our specimens, not mRNA expression; the discordance

Table 3 Correlation between PD-L1 expression in tumour cells and PD-1 expression in TILs

n=162	PD-1 expression in TILs		p=0.03
	Positive	Negative	Total
PD-L1 expression in tumours cells (no. of patients (%))			
Positive	21 (34)	40 (66)	61 (100)
Negative	19 (19)	82 (81)	101 (100)
Total	40	122	162

PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; TIL, tumour-infiltrating lymphocyte.

Table 4 Correlation between PD-L1 expression in tumours/PD-1 expression in TILs and TIL score with pCR by subtype

	TNBC			HR-/HER2+			HR+/HER2-		
	N (%)	% pCR rate	p*	N (%)	% pCR rate	p*	N (%)	% pCR rate	p*
PD-L1 expression									
positive	40 (49)	22.5 (9/40)	0.16	13 (32)	38.5 (5/13)	0.08	9 (20)	0 (0/9)	0.46
negative	41 (51)	36.6 (15/41)		28 (68)	14.3 (4/28)		35 (80)	2 (2/35)	
PD-1 expression									
positive	26 (32)	30.8 (8/26)	0.97	8 (20)	25.0 (2/8)	0.85	6 (14)	0 (0/6)	0.46
negative	56 (68)	30.3 (17/56)		32 (80)	21.9 (7/32)		36 (86)	8.3 (3/36)	
TIL score									
High	67 (73)	37.3 (25/67)	0.05	23 (55)	30.4 (7/23)	0.12	8 (17)	12.5 (1/8)	0.45
Low	25 (27)	16.0 (4/25)		19 (45)	10.5 (2/19)		38 (82)	5.3 (2/38)	

*p Values were calculated between each of the three factors and pCR by using the χ^2 exact test.

HER2, human epidermal growth factor receptor 2; HR, hormone receptors; pCR, pathological complete response; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; TIL, tumour-infiltrating lymphocytes; TNBC, triple-negative breast cancer.

between mRNA and IHC-based determination of PD-L1 expression might influence our results.^{20 32} Another reason may be attributed to the fact that upregulated PD-L1 and PD-1 were not only poor prognostic markers but, paradoxically, good predictive markers of pCR.

Therefore, the survival outcomes may have been obscured by these competing measures.

In summary, we showed that expression of PD-L1 and PD-1 is associated with higher TIL scores and pCR; conversely, expression of these proteins correlated with

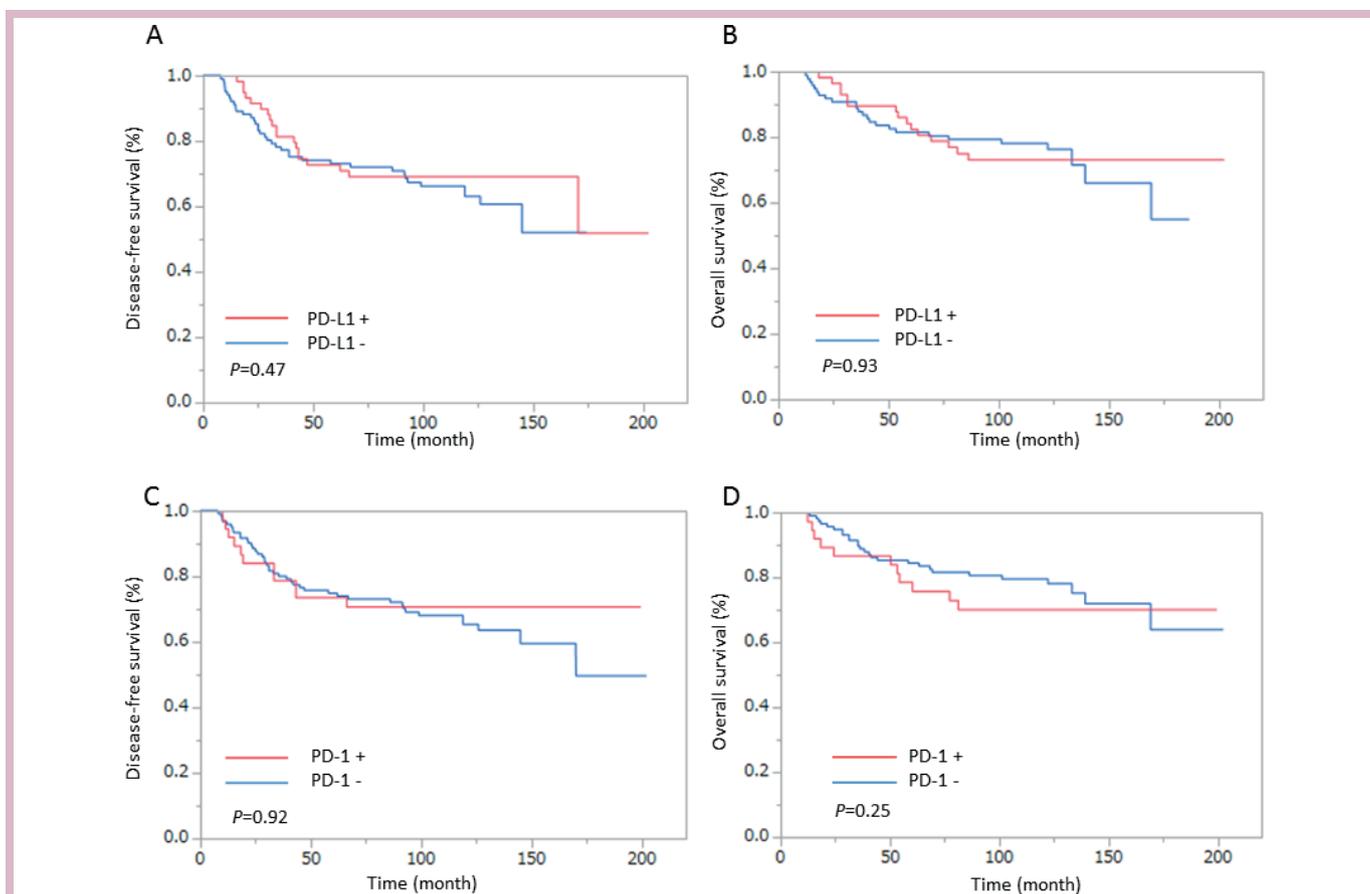


Figure 2 Survival outcomes according to PD-L1 or PD-1 expression. Kaplan-Meier estimates of disease-free survival and overall survival according to expression levels of PD-L1 and PD-1. Expression of PD-L1 and PD-1 did not significantly influence survival. (A) Disease-free survival according to PD-L1 expression. (B) Overall survival according to PD-L1 expression. (C) Disease-free survival according to PD-1 expression. (D) Overall survival according to PD-1 expression. PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1.

poor prognostic clinicopathological factors such as tumour grade and subtype. PD-L1 expression in tumours and PD-1 expression in TILs were significantly correlated, and 34% of all tumours concurrently expressed both proteins. However, our results did not support the notion that PD-L1 and/or PD-1 expression are predictive markers for survival, which had previously been shown in a study of mRNA PD-L1 expression levels in TNBC.^{21–32} The main strengths of our study were that both PD-L1 expression in tumours and PD-1 expression in TILs were evaluated in the same tumour specimens and that we were able to perform statistical analysis of PD-L1 and PD-1 expression in this context.

Our study has a number of limitations. One is its retrospective nature, as it was performed using archived specimens. A second limitation is that PD-L1 and PD-1 expression levels were evaluated by IHC and not transcriptionally. A third limitation is that our patients had received neoadjuvant chemotherapy using cytotoxic regimens, not immune checkpoint inhibitors. Namely, PD-L1 or PD-1 expression in this study does not reflect immune checkpoint inhibitor efficacy. Our data indicate that expression levels of PD-L1 and/or PD-1 are not sufficient predictive factors for survival; hence, alternative frameworks that include PD-L1, PD-1 and TILs combined with other microenvironmental factors are required for reliable prognostication.

Acknowledgements We thank to Mrs Sachiko Miura and Mrs Chizu Kina for their excellent technical assistance.

Funding This study was supported by Japan and National Cancer Center Research and Development Funds (26-A-8).

Competing interests None declared.

Ethics approval This article does not contain any studies with animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Provenance and peer review Not commissioned; internally peer reviewed

Open Access This is an Open Access article distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See: <http://creativecommons.org/licenses/by/4.0/>

© European Society for Medical Oncology (unless otherwise stated in the text of the article) 2017. All rights reserved. No commercial use is permitted unless otherwise expressly granted.

REFERENCES

- Ahmadzadeh M, Johnson LA, Heemskerck B, *et al.* Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 2009;114:1537–44.
- Dong H, Strome SE, Salomao DR, *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793–800.
- Iwai Y, Ishida M, Tanaka Y, *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 2002;99:12293–97.
- Reiss KA, Forde PM, Brahmer JR. Harnessing the power of the immune system via blockade of PD-1 and PD-L1: a promising new anticancer strategy. *Immunotherapy* 2014;6:459–75.
- Francisco LM, Salinas VH, Brown KE, *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 2009;206:3015–29.
- Zitvogel L, Kepp O, Kroemer G. Immune parameters affecting the efficacy of chemotherapeutic regimens. *Nat Rev Clin Oncol* 2011;8:151–60.
- Galon J, Angell HK, Bedognetti D, *et al.* The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* 2013;39:11–26.
- Denkert C, Loibl S, Noske A, *et al.* Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 2010;28:105–13.
- Ali HR, Provenzano E, Dawson SJ, *et al.* Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol* 2014;25:1536–43.
- Loi S, Sirtaine N, Piette F, *et al.* Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. *J Clin Oncol* 2013;31:860–7.
- Perez EA, Ballman KV, Tenner KS, *et al.* Association of stromal tumor-infiltrating lymphocytes with recurrence-free survival in the N9831 adjuvant trial in patients with early-stage HER2-positive breast cancer. *JAMA Oncol* 2016;2:56–64.
- Loi S, Michiels S, Salgado R, *et al.* Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol* 2014;25:1544–50.
- Ono M, Tsuda H, Shimizu C, *et al.* Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. *Breast Cancer Res Treat* 2012;132:793–805.
- Yamaguchi R, Tanaka M, Yano A, *et al.* Tumor-infiltrating lymphocytes are important pathologic predictors for neoadjuvant chemotherapy in patients with breast cancer. *Hum Pathol* 2012;43:1688–94.
- Mahmoud SM, Paish EC, Powe DG, *et al.* Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 2011;29:1949–55.
- Lee HJ, Seo JY, Ahn JH, *et al.* Tumor-associated lymphocytes predict response to neoadjuvant chemotherapy in breast cancer patients. *J Breast Cancer* 2013;16:32–9.
- Ghebeh H, Mohammed S, Al-Omar A, *et al.* The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia* 2006;8:190–8.
- Muenst S, Soysal SD, Gao F, *et al.* The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat* 2013;139:667–76.
- Nanda R, Chow LQ, Dees EC, *et al.* Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study. *J Clin Oncol* 2016;34:2460–7.
- Wimberly H, Brown JR, Schalper K, *et al.* PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol Res* 2015;3:326–32.
- Sabatier R, Finetti P, Mamessier E, *et al.* Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget* 2015;6:5449–64.
- Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. the value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991;19:403–10.
- Turner NC, Reis-Filho JS. Basal-like breast cancer and the BRCA1 phenotype. *Oncogene* 2006;25:5846–53.
- Allred DC, Harvey JM, Berardo M, *et al.* Prognostic and predictive factors in breast Cancer by immunohistochemical analysis. *Mod Pathol* 1998;11:155–68.
- Wolff AC, Hammond ME, Schwartz JN, *et al.* American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;25:118–45.
- Muenst S, Schaeferli AR, Gao F, *et al.* Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat* 2014;146:15–24.
- Guo Y, Yu P, Liu Z, *et al.* Prognostic and clinicopathological value of programmed death ligand-1 in breast cancer: a meta-analysis. *PLoS One* 2016;11:e0156323.
- Garon EB, Rizvi NA, Hui R, *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* 2015;372:2018–28.



29. Taube JM, Anders RA, Young GD, *et al.* Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 2012;4:127ra37.
30. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin Cancer Res* 2013;19:1021–34.
31. Teng MW, Ngiow SF, Ribas A, *et al.* Classifying cancers based on T-cell infiltration and PD-L1. *Cancer Res* 2015;75:2139–45.
32. Schalper KA, Velcheti V, Carvajal D, *et al.* In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. *Clin Cancer Res* 2014;20:2773–82.
33. Gatalica Z, Snyder C, Maney T, *et al.* Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. *Cancer Epidemiol Biomarkers Prev* 2014;23:2965–70.