

# Status and prognostic value of immunological biomarkers of breast cancer

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**Abstract.** The immune response to cancer serves an important role in disease progression and patient prognosis. For triple-negative breast cancer showing aggressive behavior, immunotherapy has a good efficacy because of the potent immunogenicity of this type of cancer. However, the dominant subtype, luminal human epidermal growth factor receptor-2 (HER2)-negative breast cancer, is less immunogenic. To determine whether luminal HER2-negative cancer reacts to the anticancer immune response, the present study analyzed the status and prognostic value of the principal immunological biomarkers of breast cancer, including tumor-infiltrating lymphocytes (TILs), CD8<sup>+</sup> T lymphocytes, the major histocompatibility complex and programmed cell death ligand-1 (PD-L1). The biomarkers were compared between patients with luminal HER2-negative breast cancer and those with immunogenic subtypes including triple-negative and HER2-overexpressed breast cancer. A total of 71 patients with primary breast cancer were classified into the immunogenic non-luminal (n=23) and less immunogenic luminal HER2-negative groups (n=48) based on immunogenicity. In the luminal HER2-negative group, compared with patients with low TIL levels, those with

high TIL levels were at an advanced stage of cancer (P=0.024) and showed worse relapse-free survival (P=0.057); however, the remaining biomarkers exhibited no association with cancer progression or prognosis. In the non-luminal group, patients with high TIL levels showed significantly better RFS than those with low TIL levels (P=0.014). Compared with non-luminal patients negative for PD-L1, those positive for PD-L1 exhibited better overall survival (P=0.064). Notably, TIL status was found to exhibit contrasting prognostic predictions based on immunogenicity. In conclusion, TILs are a strong candidate for prognostic prediction in breast cancer, regardless of the subtype. PD-L1 is a potential candidate for prognostic prediction in immunogenic breast cancers, but not in the luminal HER2-negative subtype.

## Introduction

Several biomarkers predict cancer progression, patient prognosis, and therapeutic efficacy in cancer. Some of the most useful biomarkers are involved in the growth or metastasis of cancers (1-4). In breast cancer, the biomarker human epidermal growth factor receptor-2 (HER2) can be used to predict both patient prognosis and anti-HER2 therapeutic efficacy (5-11). The use of trastuzumab, a HER2-targeting agent, for treating patients with HER2-overexpressed breast cancer that exhibits aggressive clinical behaviors and poor prognosis has significantly improved the prognoses of these patients; for these patients, treatment with trastuzumab has resulted in a prognosis as favorable as that of patients with luminal HER2-negative breast cancer in both postoperative and metastatic settings (11-15). However, for triple-negative (TN) breast cancer that exhibits clinical aggressiveness and lacks the expression of target molecules and biomarkers available for treatment, no efficient therapeutics have been established. Recent studies have found that the immune response plays a crucial role in the disease progression and prognosis of patients with cancer. Therefore, studies have investigated the use of immune checkpoint inhibitors targeting the programmed cell death 1-programmed cell death-ligand-1 (PD-1-PD-L1) axis in cancer treatment; this has resulted in dramatic positive

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*Abbreviations:* CD, cluster of differentiation; CTL, cytotoxic T lymphocyte; DAB, 3,3'-diaminobenzidine; HER2, human epidermal growth factor receptor-2; MHC, major histocompatibility complex; OS, overall survival; PBS, phosphate-buffered saline; PD-1, programmed cell death 1; PD-L1, programmed cell death-ligand-1; RFS, relapse-free survival; TIL, tumor-infiltrating lymphocyte; TN, triple-negative

*Key words:* biomarker, breast cancer, MHC, PD-L1, TILs, CD8<sup>+</sup> T lymphocytes

effects against immunogenic cancers (16-27). Although breast cancer is among the less immunogenic cancers (28-30), certain aggressive subtypes with typically poor prognoses, such as TN and HER2-overexpressed breast cancer, have been found to be potentially immunogenic (31-33).

Clinical trials of the therapeutic potential of immune checkpoint inhibitors, such as atezolizumab and pembrolizumab, in the treatment of advanced TN breast cancer have achieved an objective treatment response rate of 53.2-56.0%, with a significantly longer progression-free survival compared with that of placebo group patients (34-38). PD-1 and PD-L1 are molecules responsible for immune checkpoint processes. PD-L1 is expressed in various cells, including macrophages, monocytes, T cells, B cells, and tumor cells and binds to PD-1 receptors on T and B cells. PD-L1 is overexpressed in tumor cells and binds to PD-1 on cytotoxic T lymphocytes (CTLs). This initiates the lysis and apoptosis of cancer cells by CTLs. However, prolonged exposure to cancer cells can lead to CTL exhaustion, which reduces their ability to kill tumor cells (39). Inhibiting the interaction between PD-1 and PD-L1 prevents CTLs from becoming less responsive and helps them restore their cytotoxic activity against cancer cells. For the body to recognize cancer cells and ensure that CTLs attack them, immune cells should infiltrate the tumor mesenchyme. Immune cells can include antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes. Moreover, CD4<sup>+</sup> helper lymphocytes and CD8<sup>+</sup> CTLs can infiltrate the tumor mesenchyme and recognize antigenic epitopes present on major histocompatibility complex (MHC) molecules. Therefore, tumor-infiltrating lymphocytes (TILs) and MHC molecules are essential for inducing an antitumor immune response. Several studies have reported the utility of the level of TILs and CD8<sup>+</sup> T lymphocyte infiltrates, and expression of MHC and PD-1-PD-L1 axis for prognostic prediction in cancer (40-49). Cancer cells can gradually acquire the ability to evade the immune surveillance system of antitumor immune cells, thereby leading to cancer progression (50). Among these evasive tactics against antitumor immunity is the deletion of MHC class I molecules on the surface of cancer cells. This prevents the interaction of CTLs with T cell receptors, which is necessary for the recognition of the cancer cells by CTLs. Thus, MHC class I molecules also have prognostic significance (48-50).

As described above, antitumor immune responses can affect cancer progression and patient prognosis in immunogenic subtypes such as TN and HER2-overexpressed breast cancer; however, antitumor immunity is unlikely to affect luminal HER2-negative breast cancer, a dominant subtype, because of its lower immunogenicity. Indeed, compared with other cancer types, breast cancer including luminal HER2-negative breast cancer, which occurs in a majority of the population, exhibits fewer mismatch repair deficiencies and microsatellite instabilities; this is partly because breast cancer is a well-differentiated and slow-growing cancer (28,51). This results in a low production of non-self antigenic proteins during cancer progression. This low immunogenicity has been verified in a clinical trial evaluating an immune checkpoint inhibitor; in the trial, the treatment was inefficacious in patients with luminal HER2-negative breast cancer compared

with the beneficial effects observed in those with TN breast cancer (30).

In this study, we retrospectively evaluated the status and prognostic value of the immunological breast cancer biomarkers, TILs, CD8<sup>+</sup> T lymphocyte infiltrates, MHC molecules, and PD-L1. We compared these biomarkers between patients with less immunogenic luminal HER2-negative breast cancer and those with immunogenic non-luminal breast cancer including TN and non-luminal HER2-overexpressed breast cancers.

## Materials and methods

**Patients.** Seventy-one female patients with primary breast cancer who had undergone surgery such as mastectomy or partial resection for primary lesions with either axillary dissection or sentinel node biopsy from January 2010 to December 2021 at Kagawa University Hospital were included in this study. The exclusion criteria were as follows: previous invasive breast cancer or non-breast cancer within 5 years before surgery for primary breast cancer; any previous chemotherapy or endocrine therapy for cancer; any previous anti-HER2 therapy or other previous anticancer biologic therapy or immunotherapy; and concurrent serious diseases interfering with adjuvant therapy for breast cancer. The median patient age was 59 (35-85) years. At the time of surgery, 27 of the patients were in clinical stage 1, 42 were in stage 2, and 2 were in stage 3 (Table I). The cohort comprised 48 patients with luminal HER2-negative, 21 with TN, and 2 with non-luminal HER2-overexpressed breast cancer. Tissue samples of the main breast tumor obtained by either surgical resection or preoperative biopsy were examined.

**Evaluation of TIL levels.** TIL levels in patient tissue samples were evaluated. After the samples were fixed in formalin and embedded in paraffin, they were sectioned into 4- $\mu$ m-thick slices and stained in hematoxylin-eosin solution, as previously described (52). All mononuclear cells, including lymphocytes and plasma cells, were selected for the evaluation of TIL levels. Granulocytes and other polymorphonuclear leukocytes were excluded. As recommended in previous studies, stromal TIL levels were determined according to the area of stromal tissues occupied by mononuclear inflammatory cells over the total intratumoral stromal area (= % stromal TILs). The denominator used to determine the % stromal TIL level is the area of stromal tissue and not the number of stromal cells (52-54). Three representative fields of view were selected and the average of each TIL level was determined. We used a cutoff score of 10% as previously established (55). Therefore, a stromal TIL level  $\geq$ 10% was designated as a high TIL level, and that <10% was designated as a low TIL level.

**Immunohistochemistry.** Serial sections (4- $\mu$ m-thick) of formalin-fixed paraffin-embedded tissue specimens were stained via standard indirect immunoperoxidase procedures for PD-L1, CD8, and MHC class I molecules, according to the staining kit manufacturer's instructions. Briefly, each tissue section was deparaffinized in xylene and rehydrated in ethanol and distilled water. Antigen retrieval was performed

via 10 min of microwave treatment in 10 mM sodium citrate buffer (pH 6.0) for PD-L1 or 10 mM Tris/1 mM ethylenediaminetetraacetic acid (pH 9.0) for MHC class I molecules. Endogenous peroxidase activity was blocked by treatment with 3% H<sub>2</sub>O<sub>2</sub> for 10 min. After blocking in Tris-buffered saline with Tween-20 and 5% normal goat serum for 1 h at room temperature, the sections were incubated at 4°C overnight with antihuman PD-L1 monoclonal antibodies (clone: E1L3N, diluted 1:200, Cell Signaling Technology, Danvers, MA, USA; SP263, diluted 1:100, Ventana Medical Systems, Tucson, AZ, USA), which were produced by immunizing rabbits with peptides derived from the C-terminus of PD-L1 protein, anti-CD8 monoclonal antibody (clone: SP57, diluted 1:100, Ventana Medical Systems), or an anti-HLA class I monoclonal antibody (clone: EMR8-5, diluted 1:500, Hokudo Co., Ltd., Sapporo, Japan). The sections were then incubated with SignalStain boost IHC detection reagent (Cell Signaling Technology) for PD-L1 or Envision Dako ChemMate (Dako Ltd., Kyoto, Japan) for CD8 and MHC class I molecules. They were visualized using a SignalStain DAB (3,3'-diaminobenzidine) substrate kit (Cell Signaling Technology) for PD-L1 or Envision Dako ChemMate/horseradish peroxidase (HRP) DAB for CD8 and MHC class I molecules for 1 min. This was followed by counterstaining with hematoxylin. Isotype-matched control antibodies were used for immunohistochemistry. These were rabbit immunoglobulin G (IgG) monoclonal antibody (Cell Signaling Technology) for PD-L1, and mouse IgG monoclonal antibody (Dako) for CD8 and MHC class I molecules.

*Evaluation of PD-L1, MHC class I, and CD8 expression.* Serial sections of stained tumor tissues were independently examined by two researchers, including a pathologist. To compare the cellular staining intensities of PD-L1, CD8, and MHC class I molecules, cells from the serial sections were evaluated microscopically (magnification: x200). Three representative fields of view were selected and any expression of PD-L1 and MHC class I molecules was identified in 100 tumor cells per field. Cases in which the proportion of tumor cells positive for PD-L1 was  $\geq 1\%$  were considered PD-L1+ tumor cell-dominant. Cases in which the proportion of tumor cells positive for MHC class I molecules was  $\geq 80\%$  were considered MHC class I+ tumor cell-dominant, as previously reported (56). To evaluate CD8<sup>+</sup> T lymphocyte levels, we counted the number of CD8<sup>+</sup> T lymphocytes in three stroma fields of view and calculated the median number of CD8<sup>+</sup> T lymphocytes per field.

*Statistical analysis.* All statistical analyses were performed using SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA) software. For comparisons between two groups, we used the Mann-Whitney U test or the  $\chi^2$  test. The effects of clinical and demographic variables, clinical responses, and prognostic parameters on the duration of survival and risk of progression were assessed using Kaplan-Meier survival analyses and log-rank tests. A 95% confidence interval for the median of each variable was calculated using the Brookmeyer and Crowley method (57). All analyses were two-sided and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Association of clinicopathological patient variables and immunological biomarker status with cancer progression and prognosis.* The patient cohort included 48 (67.6%) patients with luminal HER2-negative, 21 (29.6%) with TN, and two (2.8%) with non-luminal HER2-overexpressed breast cancer. At the time of the study, 30 patients experienced relapsed lesions. The relapse-free survival (RFS) and overall survival (OS) rates 10 years after the primary operation were 60.3 and 78.1%, respectively (Table I). There were 50 (70.4%) patients positive for MHC expression and 43 (60.6%) with high TIL levels (Tables I and II). Microscopic images of low and high TIL levels are shown in Fig. 1A and B, respectively. Microscopic images of breast cancer tumors positive and negative for MHC expression are shown in Fig. 1C and D, respectively. Reactivity to E1L3N was observed in 14 (19.7%) patients and reactivity to SP263 in 39 (54.9%) patients (Tables I and III). Microscopic images of tumors positive and negative for E1L3N and SP263 were shown in Fig. 2. Although the sensitivity of reaction of the two monoclonal antibodies observed differed, all patients responsive to E1L3N exhibited reactivity to SP263 because both the monoclonal antibodies recognized antigenic determinants near the C-terminus of PD-L1 protein. Furthermore, MHC expression was significantly associated with tumor size ( $P=0.017$ ) and clinical stage ( $P=0.046$ ) and TIL level was significantly associated with tumor size ( $P=0.021$ ), nodal involvement ( $P=0.004$ ), and clinical stage ( $P=0.006$ ); however, neither MHC expression nor TIL level was associated with RFS or OS (Table II; Figs. 3 and 4). The number of CD8<sup>+</sup> T lymphocyte infiltrates in the tumor stroma was significantly associated with MHC and PD-L1 expression and TIL levels (Table II). Microscopic images of low and high counts of CD8<sup>+</sup> T lymphocyte infiltrates are shown in Fig. 5A and B, respectively. The proportion of patients positive for SP263 was significantly higher in patients positive for E1L3N than that in patients negative for E1L3N (E1L3N-negative and E1L3N-positive patients: 42.1 and 100%, respectively,  $P < 0.001$ , Table III) and the proportion of patients positive for E1L3N was significantly higher in patients positive for SP263 than that in patients negative for SP263 (SP263-negative and SP263-positive patients: 0 and 36.8%,  $P < 0.001$ ). Regarding the status of these immunological biomarkers, the proportion of patients with high TIL levels, the proportion of patients reactive to E1L3N, and the number of CD8<sup>+</sup> T lymphocyte infiltrates were significantly higher in the non-luminal group than in the luminal group (high TIL levels, 82.7% vs. 50.0%,  $P=0.009$ ; E1L3N positivity, 39.1% vs. 10.4%,  $P=0.005$ ; number of CD8<sup>+</sup> T lymphocyte infiltrates, 88.0 vs. 55.7,  $P=0.001$ , Table I). However, the status of these biomarkers showed no prognostic value, except for an almost but not quite significant association between E1L3N reactivity and shorter RFS (RFS rate at 10 years: 66.1 and 42.9% for patients negative and positive for E1L3N, respectively;  $P=0.052$ ; Figs. 3 and 4).

*Associations of immunological biomarker status with cancer progression and prognosis in patients with immunogenic non-luminal cancer.* The non-luminal group included 21 patients with TN and 2 with non-luminal HER2-overexpressed breast cancer. The proportion of patients positive for PD-L1 expression was significantly higher in patients positive for MHC

Table I. Clinical features and prognoses of the primary breast cancer patient cohort in this study.

Variable	All (n=71)	Non-luminal (n=23)	Luminal HER2 (-) (n=48)	P-value
Median age, years	59 (31-85)	58 (31-78)	60 (32-85)	0.681
Median tumor size, cm	2 (0.5-8.5)	2.1 (0.5-6.5)	1.7 (0.7-8.0)	0.269
N-positive, %	43.6%	60.9%	31.3%	0.018 <sup>a</sup>
Stage				
1	27	4	23	0.014 <sup>a</sup>
2	42	19	23	0.006 <sup>a</sup>
3	2	0	2	0.324
MHC-positive, %	70.4%	78.3%	66.7%	0.319
High TILs, %	60.6%	82.7%	50.0%	0.009 <sup>a</sup>
Median no. CD8 <sup>+</sup> T, %	66.0 (1.0-176.3)	88.0 (17.3-176.3)	55.7 (1.0-130.0)	0.001 <sup>a</sup>
PDL1-E1L3N (+), %	19.7%	39.1%	10.4%	0.005 <sup>a</sup>
PDL1-SP263 (+), %	54.9%	60.9%	47.9%	0.174
RFS at 10 years	60.3%	52.1%	66.7%	0.059
OS at 10 years	78.1%	60.9%	87.5%	0.038 <sup>a</sup>

<sup>a</sup>P<0.05. RFS, relapse-free survival; OS, overall survival; MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age and tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer and that of patients with each clinical stage, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test. The duration of RFS and OS was assessed using Kaplan-Meier survival analyses and log-rank tests.

Table II. Relationships between MHC and TILs status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of the patient cohort.

Variable	MHC(-) (n=21)	MHC(+) (n=50)	P-value	Low TIL (n=28)	High TIL (n=43)	P-value
Median age, years	61 (46-73)	57 (31-85)	0.286	60 (37-77)	58 (32-85)	0.861
Median tumor size, cm	1.7 (0.5-3.5)	2.1 (0.7-6.6)	0.017 <sup>a</sup>	1.5 (0.7-3.5)	2.1 (0.5-8.0)	0.021 <sup>a</sup>
N-positive, %	27.2%	45.1%	0.229	21.4%	55.6%	0.005 <sup>a</sup>
Median stage	1 (1-2)	2 (1-3)	0.046 <sup>a</sup>	1 (1-2)	2 (1-3)	0.006 <sup>a</sup>
MHC-positive, %	-	-	-	50.0%	83.7%	0.004 <sup>a</sup>
High TILs, %	31.8%	70.6%	0.004 <sup>a</sup>	-	-	-
Median no. CD8 <sup>+</sup> T	39.7 (1.0-109.7)	74.3 (27.0-176.3)	0.001 <sup>a</sup>	33.7 (1.0-74.3)	83.3 (31.0-176.3)	<0.001 <sup>a</sup>
PDL1-E1L3N (+), %	0%	27.5%	0.007 <sup>a</sup>	3.6%	30.2%	0.008 <sup>a</sup>
PDL1-SP263 (+), %	31.8%	60.8%	0.016 <sup>a</sup>	32.1%	65.1%	0.018 <sup>a</sup>

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage and tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

expression than that in patients negative for MHC expression (P=0.048 for E1L3N and P=0.019 for SP263, Table IV). There was no difference in the proportion of patients with high TIL levels between MHC status (MHC-negative and MHC-positive patients: 60.0 and 88.9%, respectively, P=0.140.) Reciprocally, the proportion of patients with MHC expression was significantly higher in patients positive for E1L3N or SP263 than that

in patients negative for PD-L1 expression (E1L3N-negative and E1L3N-positive patients: 64.3 and 100%, respectively, P=0.048; SP263-negative and SP263-positive patients: 50.0 and 93.3%, respectively, P=0.019, Table V). No association of high and low TIL levels with MHC and PD-L1 expression was observed (P=0.140 with MHC, P=0.084 for E1L3N, and P=0.069 for SP263). In the non-luminal group, compared with



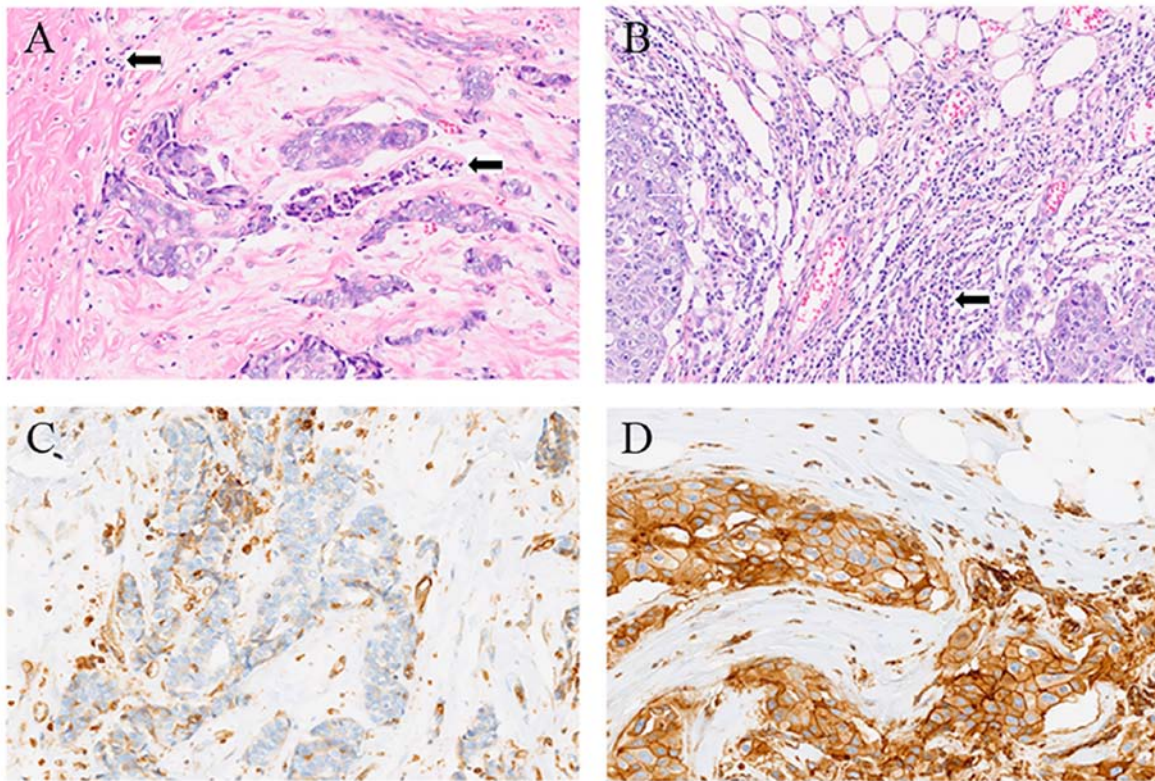


Figure 1. Stromal lymphocytic infiltrates and MHC expression in tumor cells in breast cancer tissues. (A) Low levels of TILs (<10%); (B) high levels of TILs (≥10%); (C) low MHC expression in tumor cells; (D) high MHC expression in tumor cells (magnification, x200). Arrows indicate TILs in the tumor stroma. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

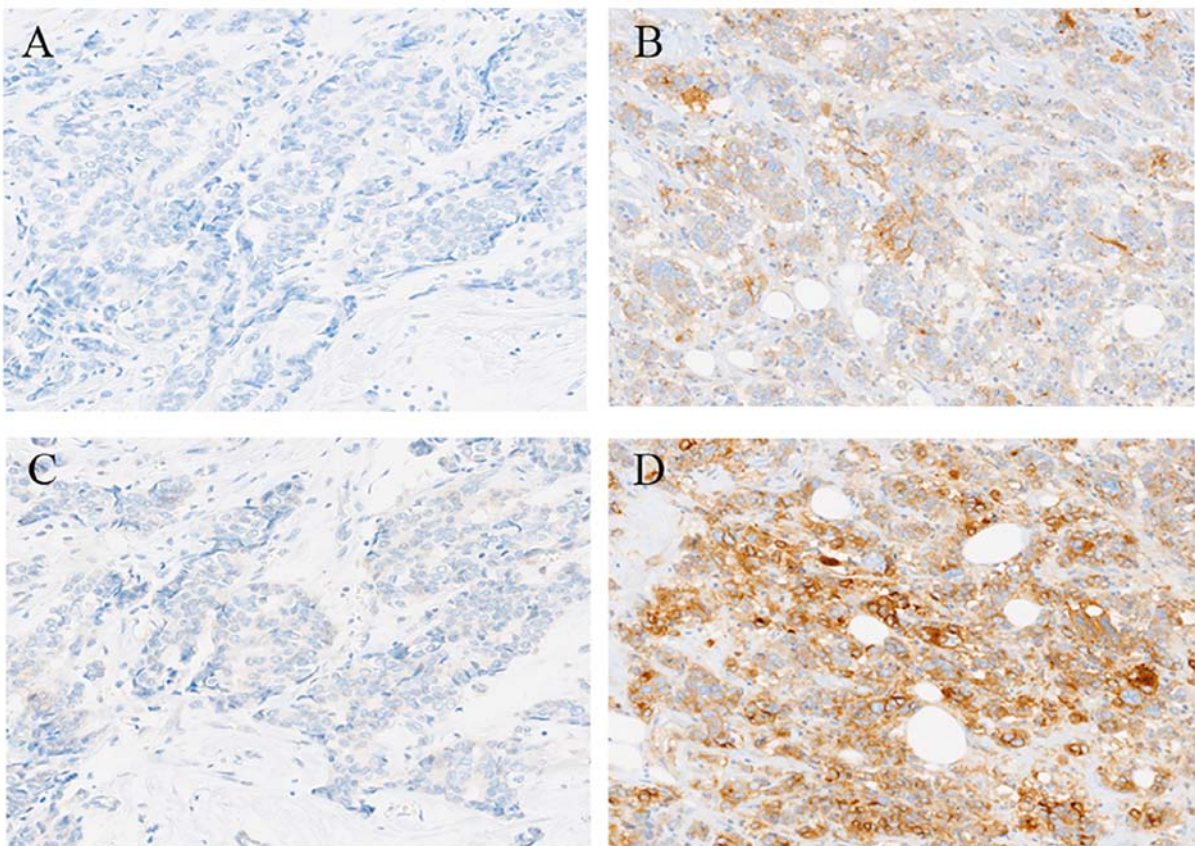


Figure 2. PD-L1 expression in breast cancer tissues. (A) None of the tumor cells showing reactivity with EIL3N; (B) many tumor cells showing reactivity with EIL3N; (C) none of the tumor cells showing reactivity with SP263; (D) many tumor cells showing reactivity with SP263 (magnification, x200). PD-L1, programmed cell death-ligand-1.

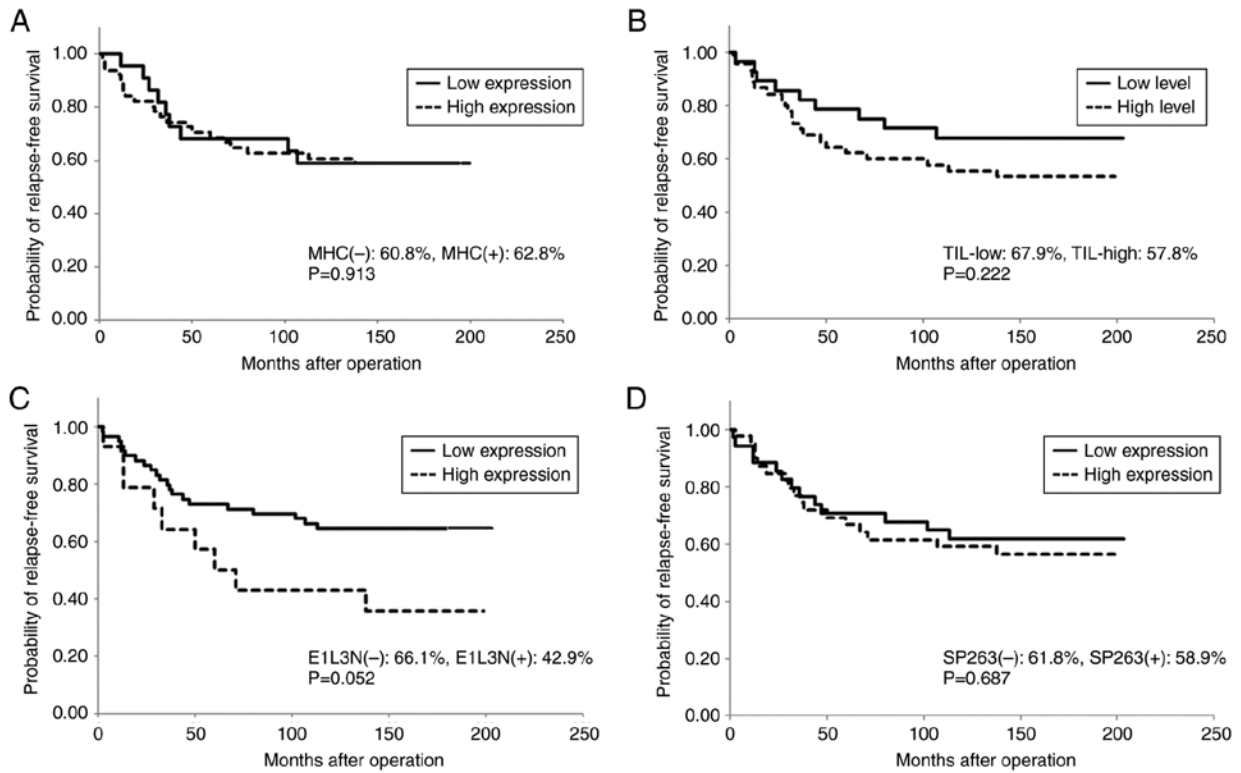


Figure 3. Comparison of relapse-free survival of two breast cancer patient groups with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with EIL3N; (D) reactivity with SP263. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

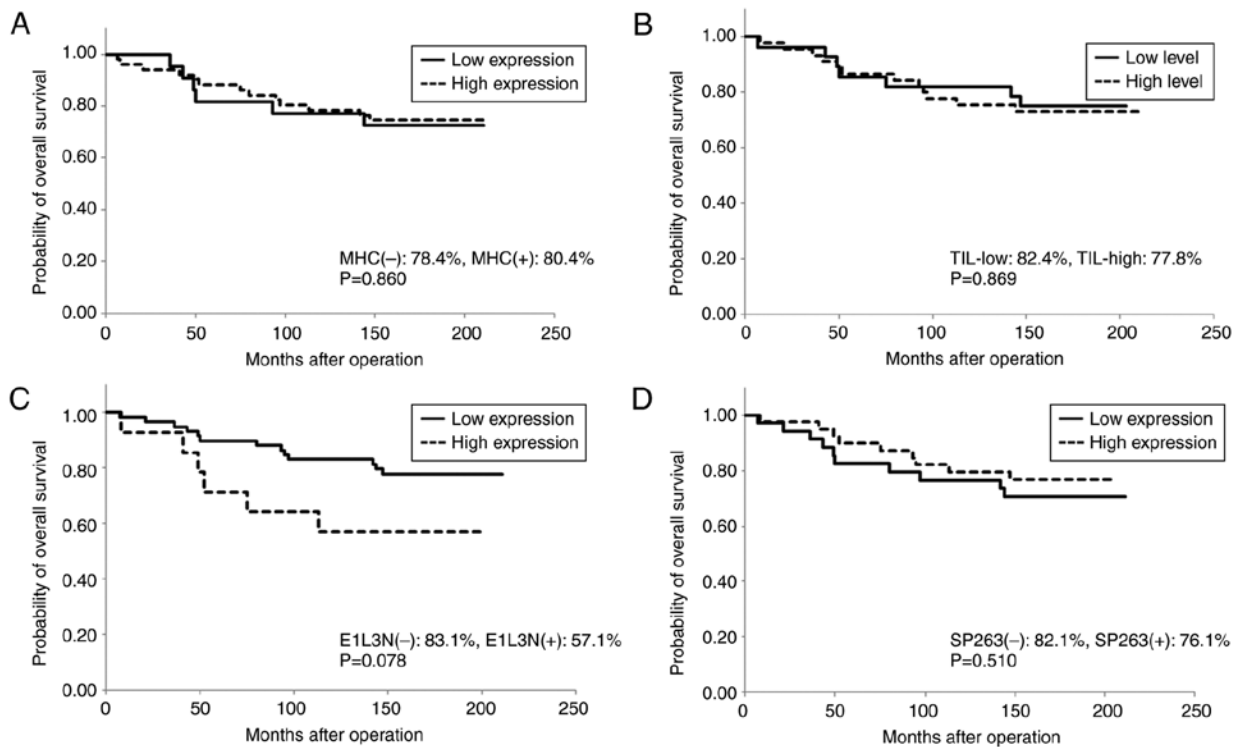


Figure 4. Comparison of overall survival of two breast cancer patient groups with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with EIL3N; (D) reactivity with SP263. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

patients with low TIL levels, patients with high TIL levels showed significantly longer RFS (low levels: median RFS of 14 months; high levels: RFS rate of 63.2% at 10 years,  $P=0.014$ ;

Fig. 6); however, TIL levels were not associated with cancer progression (Table IV). Of the remaining markers in this group, SP263 reactivity was associated with prognosis, with



Table III. Relationships between PD-L1 status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of the patient cohort.

Variable	E1L3N(-) (n=57)	E1L3N(+) (n=14)	P-value	SP263(-) (n=32)	SP263(+) (n=39)	P-value
Median age, years	60 (31-75)	57 (41-85)	0.714	62 (32-77)	58 (31-85)	0.328
Median tumor size, cm	2.0 (0.5-8.0)	2.0 (1.3-6.5)	0.624	1.9 (0.5-8.0)	2.0 (0.7-6.5)	0.766
N-positive, %	39.0%	57.1%	0.219	41.2%	41.0%	0.791
Median stage	2 (1-3)	2 (1-2)	0.553	2 (1-3)	2 (1-2)	0.705
MHC-positive, %	63.2%	100%	0.007 <sup>a</sup>	57.6%	81.6%	0.016 <sup>a</sup>
High TILs, %	52.6%	92.9	0.008 <sup>a</sup>	45.5%	73.7%	0.018 <sup>a</sup>
Median no. CD8 <sup>+</sup> T	58.3 (1.0-125.7)	100.3 (54.0-176.3)	<0.001 <sup>a</sup>	43.3 (12.0-109.7)	79.0 (1.0-176.3)	<0.001 <sup>a</sup>
PDL1-E1L3N (+), %	-	-	-	0%	36.8%	<0.001 <sup>a</sup>
PDL1-SP263 (+), %	42.1%	100%	<0.001 <sup>a</sup>	-	-	-

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage and tumor size and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

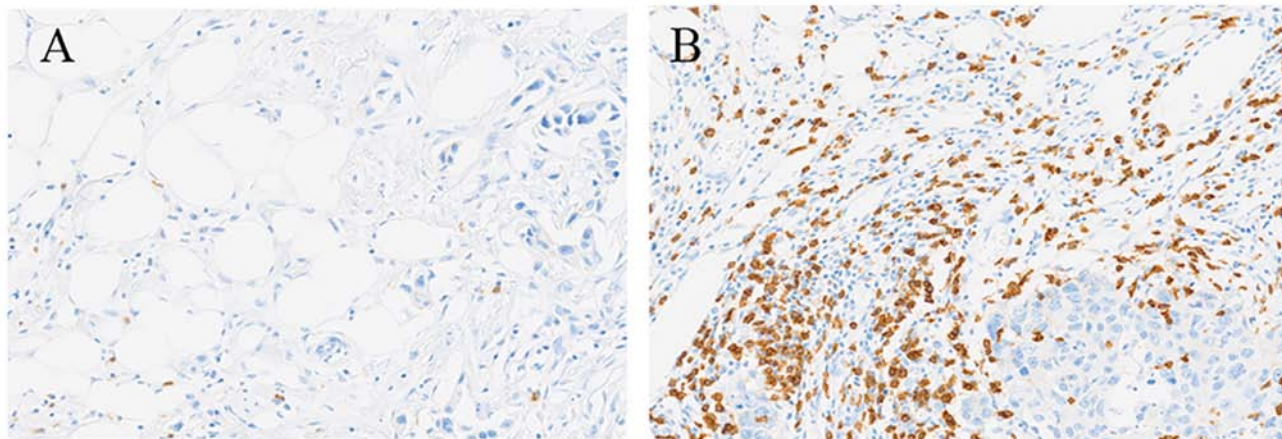


Figure 5. CD8 expression in TILs. (A) Very few CD8<sup>+</sup> T lymphocytes infiltrate in the stroma; (B) many CD8<sup>+</sup> T lymphocytes infiltrate in the stroma (magnification, x200). CD, cluster of differentiation; TILs, tumor-infiltrating lymphocytes.

reactive patients showing slightly better OS rates 10 years after their primary operation compared with nonreactive patients (37.5 and 73.3%, respectively, P=0.064; Fig. 7).

*Associations of immunological biomarker status with progression and prognosis in patients with luminal HER2-negative cancer.* In patients with luminal HER2-negative cancer, the proportion of patients positive for MHC expression and median number of CD8<sup>+</sup> T lymphocyte infiltrates per patient were significantly higher in patients with high TIL levels than those in patients with low TIL levels (MHC-positive patients: low TIL and high TIL levels, 50.0 and 83.3%, respectively, P=0.016; median CD8<sup>+</sup> T lymphocyte counts: low TIL and high TIL levels, 39.8 and 62.3, respectively, P<0.001, Table VI); moreover, a significantly higher number of patients with high TIL levels were in a more advanced stage of cancer compared with patients with low TIL levels (P=0.024). Neither MHC expression nor TIL levels showed any association with PD-L1 expression (MHC, P=0.099 for E1L3N and P=0.312 for SP263; TIL, P=0.161 for E1L3N and P=0.153 for SP263).

Furthermore, patients with high TIL levels showed a marginal trend to significance of having a shorter RFS than those with low TIL levels (RFS rate at 10 years: 79.2 and 58.3% for low and high TIL levels, respectively; P=0.057, Fig. 8). Neither MHC nor PD-L1 expression was associated with progression or prognosis in this group (Figs 8 and 9; Tables VI and VII). Remarkably, the association between high TIL levels and shorter RFS in this subtype group was contrary to that observed in the immunogenic group, in which high TIL levels were associated with longer RFS.

## Discussion

In patients with cancer, the antitumor immune response is crucial to the regulation of cancer progression and improvement of prognosis. However, cancer cells possess a wide range of mechanisms for evading host immune responses including the modification of cancer phenotypes, reduction or deletion of the expression of antigenic proteins and MHC molecules, and production of cytokines and factors that inhibit anticancer

Table IV. Relationships between MHC and TILs status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of patients with non-luminal breast cancer.

Variable	MHC(-) (n=5)	MHC(+) (n=18)	P-value	Low TIL (n=4)	High TIL (n=19)	P-value
Median age, years	66 (53-77)	57 (31-78)	0.141	47 (31-60)	61 (40-78)	0.109
Median tumor size, cm	1.8 (0.5-3.5)	2.2 (1.2-6.5)	0.359	3.3 (1.0-4.0)	2.0 (0.5-6.5)	0.545
N-positive, %	60.0%	61.1%	0.965	75.0%	57.9%	0.533
Median stage	2 (1-2)	2 (1-2)	0.310	1 (1-2)	2 (1-2)	0.750
MHC-positive, %	-	-	-	50.0%	84.2%	0.140
High TILs, %	60.0%	88.9%	0.140	-	-	-
Median no. CD8 <sup>+</sup> T	31.0 (17.3-109.7)	97.8 (32.7-176.3)	0.052	28.7 (17.3-74.3)	99.7 (51.7-176.3)	0.007 <sup>a</sup>
PDL1-E1L3N (+), %	0%	50.0%	0.048 <sup>a</sup>	0%	47.3%	0.084
PDL1-SP263 (+), %	20.0%	77.8%	0.019 <sup>a</sup>	25.0%	73.7%	0.069

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage and tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

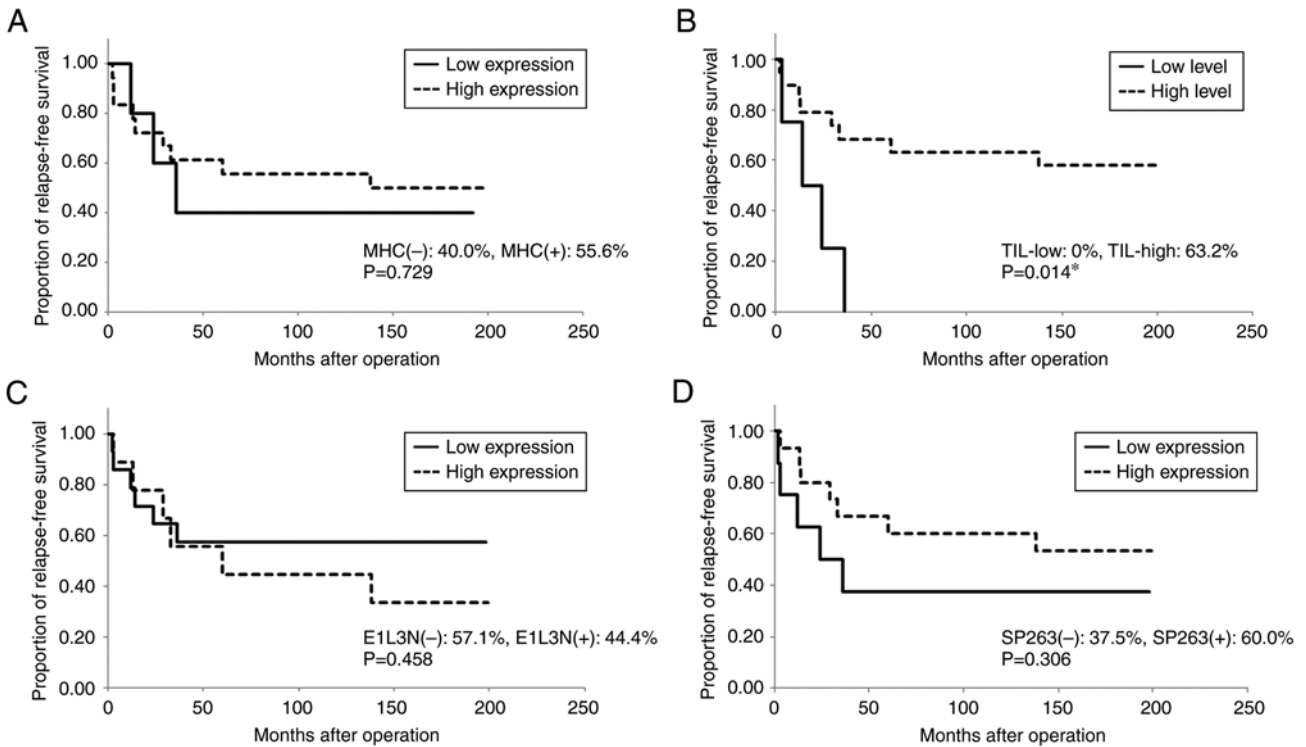


Figure 6. Comparison of relapse-free survival of two groups of patients with non-luminal immunogenic breast cancer with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with E1L3N; (D) reactivity with SP263. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

immune response activation (28,58-65). Recently, immune checkpoint inhibitors that bind to PD-1- or PD-L1-inactivating CTLs have been found to be efficacious against reduced antitumor immunity, with the ability to restart the immune response to cancer when it slows or stops (16-27). Several clinical trials have demonstrated drastically improved prognosis in patients with cancer when immune checkpoint inhibitors are added to chemotherapeutic agents (16-22,24-27). Breast cancer is among the less immunogenic cancers and tends to be minimally affected by antitumor immunity (28,29). In

fact, TIL levels in this population are not sufficiently high to exhibit prognostic value (52,66). Nevertheless, some subtypes of breast cancer such as TN and HER2-overexpressed cancer both of which can grow rapidly and show aggressive behavior have been reported to be sensitive to antitumor immune responses and have demonstrated favorable responses to immune checkpoint inhibitors (34-38). Immunological biomarkers, including TILs, CD8<sup>+</sup> T lymphocyte infiltrates, MHC, and the PD-1-PD-L1 axis, have been found to be useful for predicting cancer progression and prognosis of patients



Table V. Relationships between PD-L1 status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of patients with non-luminal breast cancer.

Variable	E1L3N(-) (n=14)	E1L3N(+) (n=9)	P-value	SP263(-) (n=8)	SP263(+) (n=15)	P-value
Median age, years	61 (31-77)	53 (40-76)	0.250	64 (41-77)	56 (31-76)	0.125
Median tumor size, cm	2.4 (0.5-3.5)	2.0 (1.2-6.5)	0.785	2.6 (0.5-3.5)	2.1 (1.2-6.5)	0.716
N-positive, %	57.1%	66.7%	0.655	62.5%	60.0%	0.909
Median stage	2 (1-2)	2 (1-2)	0.520	2 (1-2)	2 (1-2)	0.150
MHC-positive, %	64.3%	100%	0.048 <sup>a</sup>	50.0%	93.3%	0.019 <sup>a</sup>
High TILs, %	71.4%	100%	0.084	62.5%	93.3%	0.069
Median no. CD8 <sup>+</sup> T	75.3	101.0	0.012 <sup>a</sup>	42.2	101.0	0.004 <sup>a</sup>
	(17.3-125.7)	(71.7-176.3)		(17.3-109.7)	(71.7-176.3)	
PDL1-E1L3N (+), %	-	-	-	0%	60.0%	0.006 <sup>a</sup>
PDL1-SP263 (+), %	42.9%	100%	0.006 <sup>a</sup>	-	-	-

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

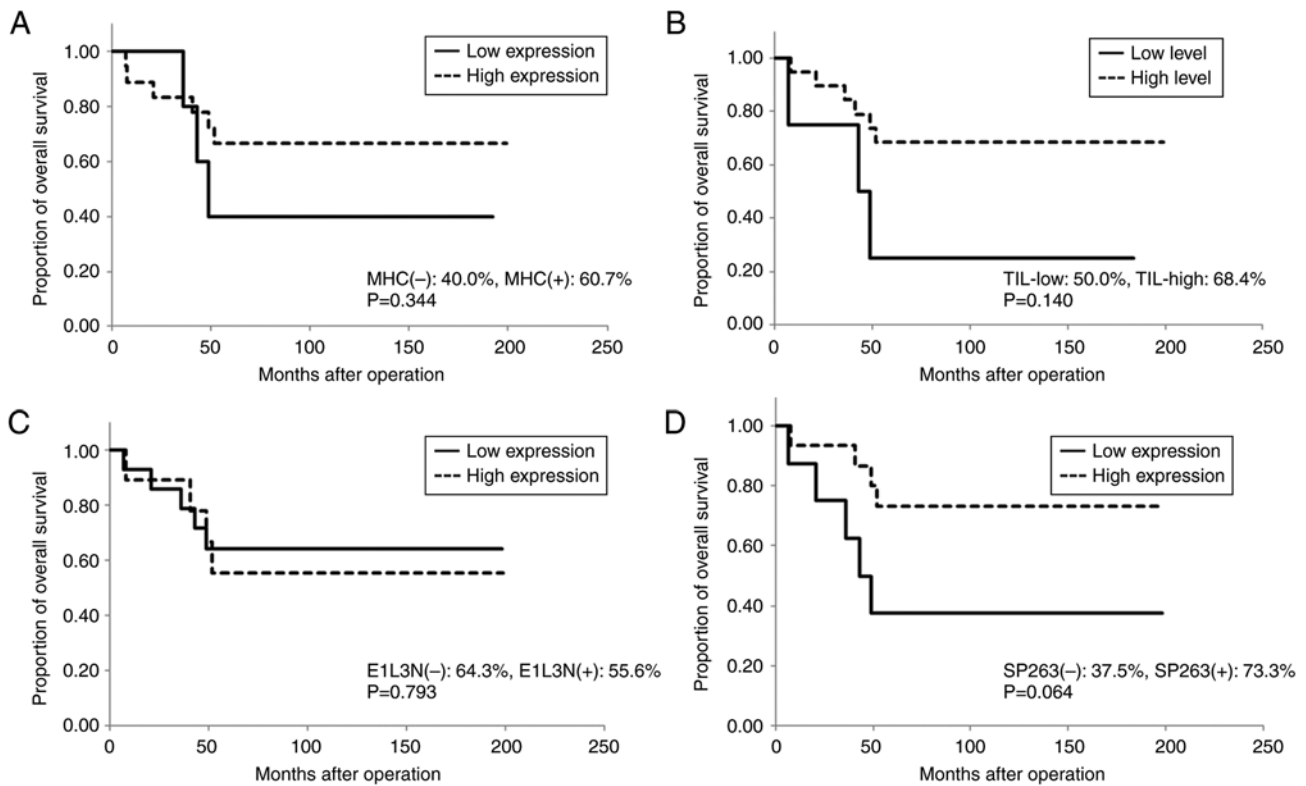


Figure 7. Comparison of overall survival of two groups of patients with non-luminal breast cancer with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with E1L3N; (D) reactivity with SP263. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

with the immunogenic subtypes of breast cancer (40-49). However, these markers have been found to predict different prognostic outcomes (35,40-42,67) owing to differences in patient backgrounds and disease stage as well as the proportion of each subtype and combination of biomarkers studied. Furthermore, no report has analyzed associations of cancer progression and patient prognosis with the four principal immunological biomarkers simultaneously in each subtype of breast cancer. Therefore, although immunological biomarkers

are expected to be useful in breast cancer, it remains unclear whether they have any prognostic value, particularly in luminal HER2-negative breast cancer.

We evaluated the status of immunological biomarkers, including TIL levels, CD8<sup>+</sup> T lymphocyte infiltrate count, MHC expression, and PD-L1 expression, in the tumors of 71 patients with primary breast cancer to determine their utility as predictors of cancer progression and prognosis. To date, only B cells and macrophages in TILs have been found

Table VI. Relationships between MHC and TILs status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of patients with luminal HER2-negative breast cancer.

Variable	MHC(-) (n=16)	MHC(+) (n=32)	P-value	Low TIL (n=24)	High TIL (n=24)	P-value
Median age, years	61 (46-73)	58 (32-85)	0.641	61 (45-77)	58 (32-85)	0.411
Median tumor size, cm	1.7 (0.7-3.1)	1.9 (0.7-8.0)	0.063	1.5 (0.7-3.1)	2.4 (0.8-8.0)	0.009 <sup>a</sup>
N-positive, %	18.8%	31.3%	0.191	12.5%	50.0%	0.006 <sup>a</sup>
Median stage	1 (1-2)	2 (1-3)	0.270	1 (1-3)	2 (1-3)	0.024 <sup>a</sup>
MHC-positive, %	-	-	-	50.0%	83.3%	0.016 <sup>a</sup>
High TILs, %	25.0%	62.5%	0.016 <sup>a</sup>	-	-	-
Median no. CD8 <sup>+</sup> T	39.7	62.3	0.002 <sup>a</sup>	39.8	62.3	<0.001 <sup>a</sup>
	(1.0-75.3)	(24.7-137.0)		(1.0-66.7)	(24.7-137.0)	
PDL1-E1L3N (+), %	0%	12.5%	0.099	4.2%	16.7%	0.161
PDL1-SP263 (+), %	37.5%	53.1%	0.313	37.5%	58.3%	0.153

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage and tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

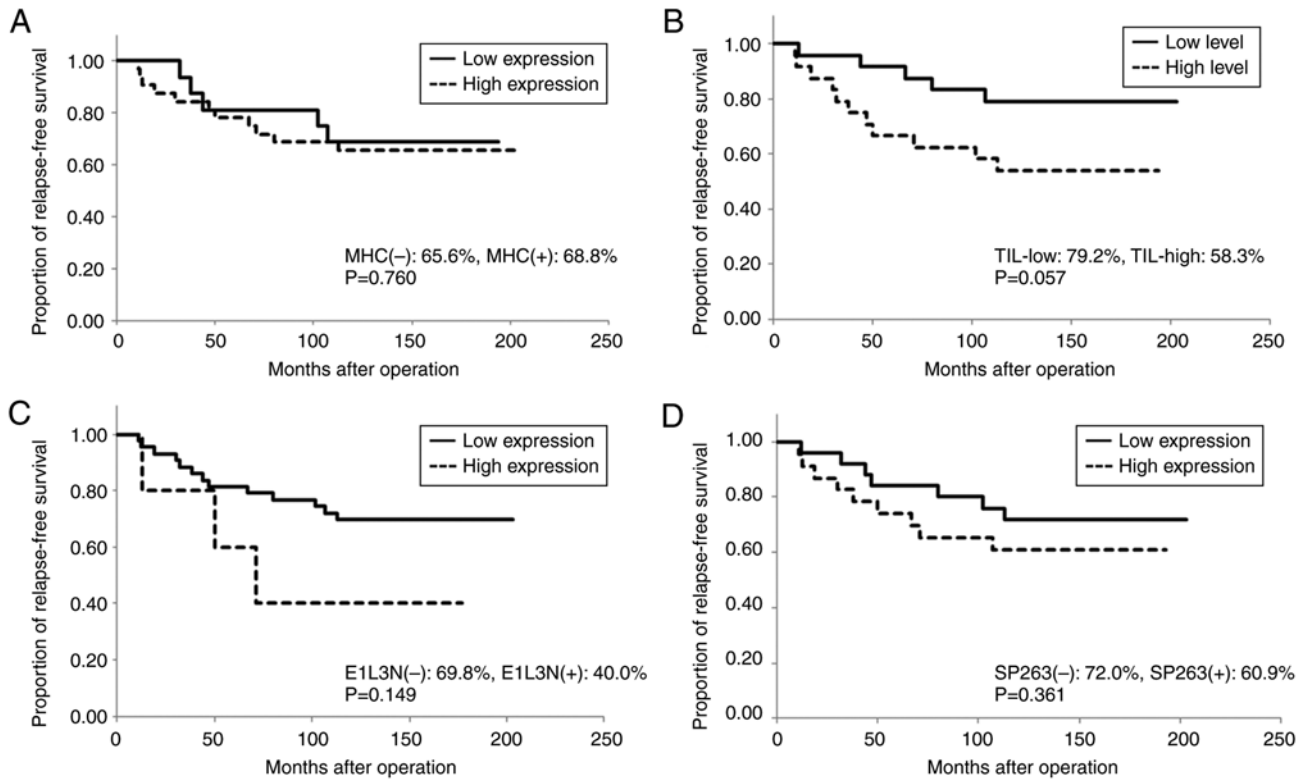


Figure 8. Comparison of relapse-free survival of two groups of patients with luminal HER2-negative breast cancer with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with E1L3N; (D) reactivity with SP263. HER2, human epidermal growth factor receptor-2; MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

to predict survival rates in luminal HER2-negative breast cancer (52); to the best of our knowledge, the prognostic value of other biomarkers has not been previously evaluated in this population. This is the first report on the status and prognostic value of principal immunological biomarkers such as MHC, TILs, CD8<sup>+</sup> T lymphocyte infiltrates, and PD-L1 in luminal HER2-negative and other breast cancer subtypes. Monoclonal antibodies against PD-1 or PD-L1 (SP142 and 22C3) currently

available as a companion diagnostic agent for potential breast cancer treatment with immune checkpoint inhibitors have demonstrated different prognostic capacities (37-41,67). These monoclonal antibodies exhibited positivity in <5% of patients with luminal HER2-negative breast cancer in our preliminary study (data not shown). Therefore, in the present study, we used alternative clones (E1L3N and SP263) available for use with non-small cell lung cancer (35,56,68-70).

Table VII. Relationships between PD-L1 status of breast cancer and cancer progression and prognosis as determined by the clinicopathological feature of patients with luminal HER2-negative breast cancer.

Variable	E1L3N(-) (n=43)	E1L3N(+) (n=5)	P-value	SP263(-) (n=25)	SP263(+) (n=23)	P-value
Median age, years	59 (32-77)	63 (53-85)	0.354	62 (32-77)	59 (38-85)	0.904
Median tumor size, cm	1.7 (0.5-3.5)	1.5 (1.3-4.0)	0.907	1.7 (0.7-8.0)	1.5 (0.7-4.0)	0.329
N-positive, %	27.9%	40.0%	0.659	36.0%	26.1%	0.464
Median stage	2 (1-3)	1 (1-2)	0.517	2 (1-3)	1 (1-2)	0.336
MHC-positive, %	62.8%	100%	0.098	60.0%	73.9%	0.312
%High TILs, %	46.5%	80.0%	0.161	40.0%	60.8%	0.153
Median no. CD8 <sup>+</sup> T	53.3 (1.0-108.0)	80.0 (54.0-130.0)	0.097	42.0 (12.0-120.3)	66.0 (1.0-130.0)	0.021 <sup>a</sup>
PDL1-E1L3N (+), %	-	-	-	0%	21.7%	0.015 <sup>a</sup>
PDL1-SP263 (+), %	41.9%	100%	0.015 <sup>a</sup>	-	-	-

<sup>a</sup>P<0.05. MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes. Median age, stage and tumor size, and the number of CD8<sup>+</sup> T lymphocytes were compared between two groups using Mann-Whitney-U test. Positive rates of MHC and PD-L1 expression, the proportion of patients with N-positive cancer, and high TIL rates were compared between two groups using  $\chi^2$  test or Fisher's exact test.

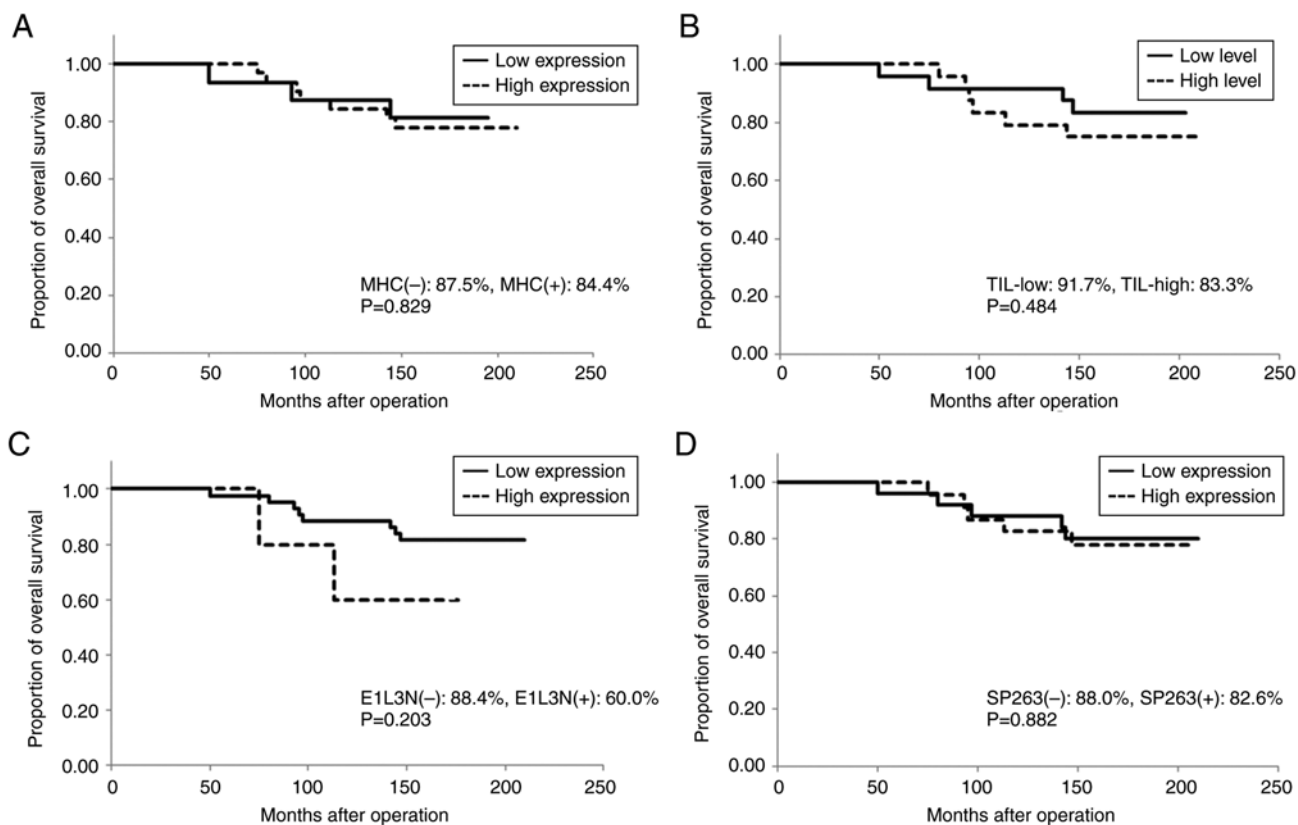


Figure 9. Comparison of overall survival of two groups of patients with luminal HER2-negative breast cancer with different biomarker statuses. (A) MHC expression; (B) TIL levels; (C) reactivity with E1L3N; (D) reactivity with SP263. HER2, human epidermal growth factor receptor-2; MHC, major histocompatibility complex; TILs, tumor-infiltrating lymphocytes.

We observed that PD-L1 expression (reactive with both E1L3N and SP263) was generally associated with MHC expression in tumor cells and with stromal TIL levels (Table III). We further found that CD8<sup>+</sup> T lymphocyte infiltrate counts were significantly associated with the status of all biomarkers in all breast cancer subtypes. These results suggest that according to breast cancer subtype, CD8<sup>+</sup> CTLs

in stromal TILs can recognize tumor antigens to varying degrees in an MHC-restricted manner and lyse cancer cells. Furthermore, the CTL response to breast cancer can be inactivated by PD-1-PD-L1 interaction.

The present study results suggested that MHC and TIL status were strongly associated with cancer progression and that PD-L1 status (in terms of its reactivity with E1L3N)

exhibited possible prognostic value for all breast cancer subtypes. To determine whether the status of these biomarkers differed between cancer subtypes and whether they interacted with cancer progression or prognosis in each subtype, the patients were classified into two cancer subtype groups according to immunogenicity: patients with less immunogenic luminal HER2-negative cancer and those with immunogenic non-luminal breast cancers. In the immunogenic group, no association was observed between TIL levels and MHC expression; however, TIL levels and MHC expression were closely associated with PD-L1 expression (Tables IV and V). Of the patients who tested negative for MHC expression, those reactive to E1L3N and SP263 accounted for only 0 and 20% (1 case), respectively (Table IV). Similarly, the number of patients reactive to E1L3N and SP263 among those with low TIL levels was quite low (no case and 1 case, respectively). Indeed, there is a small population who is deficient in biomarker expression in immunogenic breast cancer subtypes. These patients are unlikely to be affected by antitumor immunity. Furthermore, TIL status was found to be a good predictor of prognosis in this group, with high TIL levels indicating significantly longer RFS. These results are consistent with those of previous studies in that high TIL levels were associated with good prognoses in TN and HER2-overexpressed breast cancer (52,66). Moreover, high SP263 reactivity was associated with longer OS (Fig. 7D). The close relationship of PD-L1 expression with good patient prognosis has previously been reported in several studies (40–42,67), and our results are consistent with those findings. Therefore, TIL levels and PD-L1 expression can be useful prognostic biomarkers in immunogenic breast cancer. However, contrary to our expectations, none of the biomarkers was associated with cancer progression in this subtype. It is possible that antitumor immunity is merely one of the factors influencing cancer progression. Tumor characteristics such as growth ability, differentiation grade, and metastatic ability, are also likely to considerably contribute to cancer progression.

In the less immunogenic luminal HER2-negative breast cancer group, high TIL levels were strongly associated with cancer progression and associated with poor prognoses (Table VI and Fig. 8B). In the luminal group, among patients negative for MHC expression or those with low TIL levels, only few patients exhibited reactivity to both anti-PD-L1 monoclonal antibodies (Table VI). Therefore, it is difficult to arrive at a conclusion from the data of patients who tested negative for biomarker expression. The other biomarkers showed no association with either cancer progression or prognosis. Remarkably, the relationship between high TIL levels and poor prognosis in the luminal HER2-negative group was contrary to that observed in the immunogenic non-luminal group, in which high TIL levels were associated with good prognoses. This suggests that in the immunogenic non-luminal population, TILs in the tumor stroma contribute to the immunosuppression of cancer, thereby prolonging RFS. Conversely, the lower level of immunogenicity in luminal HER2-negative tumor cells reduces their receptivity to host immune responses, thus allowing more aggressive growth and progression. Based on the relationship observed between high TIL levels and poor patient prognosis as well as the significantly lower proportion of patients with PD-L1 expression or those with high TIL levels in the luminal group compared with those in the non-luminal

group, we confirmed that luminal HER2-negative breast cancer is less immunogenic. Therefore, TIL status in different breast cancer subtypes appears to reflect the distinct microbiology of tumor cells of the given subtype, in terms of the marked difference in their susceptibility to host immune responses. In the literature, only two studies have reported the relationship between TIL levels and patient prognosis in luminal HER2-negative breast cancer. Denkert *et al* (52) reported significant correlations between high TIL levels and shorter OS. The result was consistent with the findings of the present study. However, the other study reported no significant relationship between them (66). In both studies, good correlations were observed between high TIL levels and favorable patient prognoses in TN and HER2-overexpressed breast cancer subtypes. To clarify the association between TIL levels and prognosis, further studies including larger cohorts of patients with luminal HER2-negative cancer are required; these studies should aim to perform a detailed analysis for determining the lymphocyte and antigen-presenting cell populations that infiltrate the tumor stroma and the specific cytokines (e.g., interferon-gamma or tumor growth factor-beta) responsible for immune activation.

One of the limitations of our study is the small sample size ( $n=71$ ); we thus could not classify a sufficient number of patients into groups to perform more convincing comparative analyses. Furthermore, we did not analyze systemic immunological responses, such as leukocyte profiles in peripheral blood, immunoglobulin and complement levels, or cytokine production in the studied patients. By including analysis of systemic immunological responses in patients with breast cancer in a future study, we will be able to understand the role of antitumor immunity more comprehensively in breast cancer. In this study, we used two anti-PD-L1 monoclonal antibodies, which were produced by immunizing rabbits with synthetic peptides derived from residues near the C-terminus of PD-L1 protein. The sensitivity of SP263 in detecting PD-L1 expression was generally higher than that of E1L3N. Although the precise epitopes of both monoclonal antibodies has not been reported, these may be different but located nearby. As these antibodies recognize their antigenic determinants on the three-dimensional components of the target protein in immunological assays, the sensitivity of each monoclonal antibody is expected to differ. Regarding the prognostic value of the PD-L1 status in luminal HER2-negative breast cancer, Zhang *et al* (67) found no association between PD-L1 expression and OS; this finding was consistent with our results. However, significant associations were observed between PD-L1 expression and survival rates in TN and HER2-overexpressed breast cancer subtypes.

In conclusion, the immunological biomarkers MHC, TIL, and PD-L1 exhibited different patterns of expression depending on the breast cancer subtype of the patient. However, CD8<sup>+</sup> T lymphocyte infiltrate counts were closely associated with TIL levels and MHC and PD-L1 expression regardless of the breast cancer subtype. Of these biomarkers, only TIL levels are expected to be associated with cancer progression and patient prognosis, regardless of the breast cancer subtype. Although the PD-L1 protein reactive to SP263 is a potential prognostic biomarker in immunogenic cancers, it is unrelated to either cancer progression or patient prognosis in luminal HER2-negative breast cancer.



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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

CM, KeK and KT conceived and designed the present study. KaK, SN, SH, MM and TM contributed to data acquisition and analysis. KeK, CM, TY and RH were major contributors in writing the manuscript. TY RH and NH were involved in data interpretation and discussion. NA and MI performed the statistical analysis. NH, NA and MI confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The research protocol for this study complied with the guidelines of the Ethics Committee at Kagawa University Hospital and was approved by the ethical review board of Kagawa University (approval no. HEISEI23-085); it conformed to the provisions in the Declaration of Helsinki in 1995. Written informed consent to participate was obtained from all study participants.

## Patient consent for publication

When patients were given written information about the present study, written patient consent for publication was also obtained.

## Competing interests

The authors declare that they have no competing interests.

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