Phospho-STAT1 expression as a potential biomarker for anti-PD-1/anti-PD-L1 immunotherapy for breast cancer

YUKO NAKAYAMA1,2, KOSAKU MIMURA2-5, TOMOAKI TAMAKI6, KENSUKE SHIRAISHI1, LEY-FANG KUA7, VIVIEN KOH7, MASATO OHMORI1, AYAKO KIMURA1, SHINGO INOUE1, HIROKAZU OKAYAMA2, YOSHIYUKI SUZUKI8, TADAO NAKAZAWA8, DAISUKE ICHIKAWA1 and KOJI KONO2

1First Department of Surgery, University of Yamanashi, Yamanashi 409-3898; Departments of 2Gastrointestinal Tract Surgery, 3Blood Transfusion and Transplantation Immunology, 4Advanced Cancer Immunotherapy, 5Progressive DOHaD Research, and 6Radiation Oncology, Fukushima Medical University School of Medicine, Fukushima, 960-1295, Japan; 7National University Cancer Institute, Singapore, National University Health System, Singapore 119228, Republic of Singapore; 8Department of Pathology, University of Yamanashi, Yamanashi 409-3898, Japan

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Abstract. In the present study, we evaluated the mechanisms of programmed death ligand 1 (PD-L1) expression in the breast cancer microenvironment, focusing on the role of interferon-γ (IFN-γ), and the clinical indications for anti-programmed cell death 1 (PD-1) /anti-PD-L1 immunotherapy. We evaluated PD-L1 expression in 4 breast cancer cell lines in the presence of 3 types of inhibitors, as well as IFN-γ. The expression of phosphorylated signal transducer and activator of transcription 1 (p-STAT1), one of the IFN-γ signaling pathway molecules, was analyzed using immunohistochemistry (IHC) in relation to PD-L1 and human leukocyte antigen (HLA) class I expression on cancer cells and tumor-infiltrating CD8-positive T cells in 111 patients with stage II/III breast cancer. Using The Cancer Genome Atlas (TCGA) database, the correlation of the IFN-γ signature with PD-L1 expression was analyzed in breast invasive carcinoma tissues. As a result, the JAK/STAT pathway via IFN-γ was mainly involved in PD-L1 expression in the cell lines examined. IHC analysis revealed that the PD-L1 and HLA class I expression levels were significantly upregulated in the p-STAT1-positive cases. TCGA analysis indicated that the PD-L1 expression and IFN-γ signature exhibited a positive correlation. On the whole, these findings suggest that PD-L1 and HLA class I are co-expressed in p-STAT1-positive breast cancer cells induced by IFN-γ secreted from tumor infiltrating immune cells, and that p-STAT1 expression may be a potential biomarker for patient selection for immunotherapy with anti-PD-1/anti-PD-L1 monoclonal antibodies.

Introduction

Breast cancer is the most common type cancer affecting women, and has an increasing worldwide incidence (1,2). Breast cancer is divided into 5 subtypes, according to immunohistological and genetic characteristics (3), and the selection of therapeutic interventions, such as surgery, radiotherapy, hormonal therapy, chemotherapy, molecular-targeted therapy or combinations thereof, are dependent on these subtypes (4). However, for aggressive breast cancer phenotypes, such as triple-negative breast cancer, the improvement of treatment strategies and the development of novel therapies are warranted for more effective treatment.

Recently, the importance of immune checkpoints in the tumor microenvironment has been elucidated, and the anti-tumor effect of immune checkpoint inhibitors (ICIs) has been reported in various types of cancer (5). Anti-programmed cell death 1 (PD-1)/anti-programmed death ligand 1 (PD-L1) monoclonal antibodies (mAbs) have been shown to be effective in several types of cancer, including lung, gastric and kidney cancer (6-8). In addition, the outcomes of several clinical trials with ICIs for patients with breast cancer have been reported (9-11). As such, issues regarding patient selection and biomarkers to predict responses to ICIs in breast cancer patients remain debatable, and urgently need to be clarified (9-11).
It has recently been reported that conditions within the tumor microenvironment that are suitable for immunotherapy with anti-PD-1/anti-PD-L1 mAbs include the following: The presence of cytotoxic T lymphocytes (CTLs), the expression of human leukocyte antigen (HLA) class I on the tumor cells, and a significant load of neo-antigens and the expression of PD-L1 on tumor cells (12-15). Previously, we reported that HLA class I and PD-L1 were mainly upregulated by interferon-γ (IFN-γ) produced by CD8-positive T lymphocytes within the tumor microenvironment in gastric cancer (14). In the present study, using breast cancer cell lines and clinical samples, we investigated the mechanisms through which PD-L1 expression is regulated within the tumor microenvironment in breast cancer, paying particular attention to IFN-γ regulation, and discussed the clinical implications of anti-PD-1/anti-PD-L1 mAbs in breast cancer patients.

Materials and methods

Breast cancer cell lines and in vitro drug treatment. A total of 4 breast cancer cell lines, MRK-nu-1, BT-549, MCF-7 and MDA-MB-231, were used in the current study. MRK-nu-1 was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The BT-549, MCF-7 and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All the cell lines, which confirmed the absence of mycoplasma, were cultured in RPMI-1640 containing L-glutamine (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) with 5% fetal calf serum (Invitrogen/Thermo Fisher Scientific) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and were verified as authentic through a short tandem repeat analysis. The following reagents were used in in vitro drug treatment: IFN-γ (R&D Systems, Minneapolis, MN, USA), the ras/mitogen-activated protein kinase (MAPK) inhibitor, PD98059 (Cell Signaling Technology, Danvers, MA, USA), the phosphatidylinositol-3-kinase-protein kinase B (PI3K/AKT) inhibitor, wortmannin (Cell Signaling Technology), and lapatinib (GlaxoSmithKline, Brentford, UK). Lapatinib has been reported to be a combined epidermal growth factor receptor/human epidermal growth factor receptor 2 tyrosine kinase inhibitor and can inhibit both the MAPK and PI3K/AKT pathways (13). DMSO (Sigma-Aldrich) was used as a vehicle and a negative control for all treatments, and the cells were cultured and treated in 12-well plates (Thermo Fisher Scientific).

Clinical samples. Surgically-resected specimens were obtained from 111 patients who had undergone surgery for breast cancer at the First Department of Surgery at Yamanashi University (Yamanashi, Japan) between 2010 and 2014. Patients with stage II or III disease who required adjuvant chemotherapy were enrolled into the study. No patients had received pre-operative anti-tumor therapies, such as radiotherapy or chemotherapy. Clinical and pathological information were retrospectively obtained by reviewing the medical records. Tumor grade and stage were defined in accordance with the UICC TNM classification (7th edition) (16), and the histological classification was defined in accordance with the criteria of the Japanese Breast Cancer Society (17th edition) (17). This study was conducted in accordance with the Declaration of Helsinki, and was approved by the Institutional Ethical Committee of Yamanashi University (Reference 1622). Written informed consent was obtained from all participants.

Flow cytometry. The cells were stained with antibodies as previously described (13), and the following antibodies were used for staining: Annexin V-FITC (556420; BD Biosciences, San Jose, CA, USA) at 1:20; 7-Aminoactinomycin D (559925; BD Biosciences) at 1:20, and anti-human CD274 (PD-L1) PE (12-5983-42; eBioscience, Santa Clara, CA, USA) at 1:20. An isotype-matched immunoglobulin served as a negative control, and dead and/or apoptotic cells were excluded using Annexin V and 7-Aminoactinomycin D. Staining was detected using an LSRII flow cytometer (BD Biosciences).

Western blot analysis. All the samples were prepared, stained with the antibodies and visualized as previously described (18). The following primary antibodies were purchased from Cell Signaling Technology: STAT1 (14994S) at 1:1,000, p-STAT1 (8826S) at 1:1,000, p44 MAPK (ERK1/2) (4695S) at 1:1,000, phospho-p44 MAPK (p-ERK1/2) (4370S) at 1:2,000 and β-actin antibodies (4970S) at 1:2,000. A horseradish peroxidase-linked anti-rabbit IgG (7074; Cell Signaling Technology) at 1:2,000 was used as the secondary antibody.

Gene expression microarray analysis for drug-treated cell lines. The isolation of total RNA and microarray gene expression analysis were performed as previously described (14).

IHC staining. Four-micron-thick sections were deparaffinized and rehydrated. The slides were incubated with epitope retrieval solution (Agilent Technologies, Santa Clara, CA, USA) at varying conditions for different proteins: CD8, pH 9.0 for 20 min at 95-99°C in a water bath; p-STAT1, pH 6.0 for 10 min at 95-99°C in a water bath; HLA class I-A, B and C, pH 6.0 for 20 min at 121°C in an autoclave; PD-L1, pH 6.0 for 10 min at 110°C in an autoclave. All slides were incubated with peroxidase blocking solution (Agilent Technologies) for 10 min. Thereafter, the slides were incubated at 37°C for 60 min or 4°C overnight with the following primary antibodies: CD8 (clone C8/144B, M7103; Agilent Technologies) at 1:100; p-STAT1 (clone D3B7, 8826S; Cell Signaling Technology) at 1:800; HLA class I-A, B and C (clone EMR8-5, AB-46; Hokudo, Sapporo, Japan) at 1:200; and PD-L1 (clone 28-8, ab205921; Abcam, Cambridge, UK) at 1:400. Subsequently, for CD8, HLA class I and PD-L1, the slides were incubated with an avidin-biotinylated enzyme complex (Vector Laboratories, Burlingame, CA, USA), whereas the slides for p-STAT1 were incubated with a horseradish peroxidase-coupled anti-rabbit polymer (8114; Cell Signaling Technology), which was a ready-to-use solution. The slides were then incubated with diamobenzidine (Agilent Technologies) at room temperature for 5 min and counterstained with Mayer’s hematoxylin solution, (131-09665; Wako/ Fujifilm, Tokyo, Japan) at room temperature for 1 min.

Assessment of IHC staining. IHC analysis was performed by two independent observers (TN and YN), who were blinded to all of the clinical data. For assessment of CD8,
PD-L1, HLA class I and p-STAT1, hotspot areas of tumor infiltrating lymphocytes were reviewed in 4 randomly selected independent areas in marginal tumor regions at x400 magnification. The expression levels were evaluated based on IHC staining in 4 independent areas. CD8 was defined as the number of stained lymphocytes, and calculated as the mean value of the 4 areas. p-STAT1 staining was evaluated via nuclei staining in the tumor cells and tumor infiltrating immune cells (TIICs). The HLA class I staining intensity was evaluated by the following criteria: Positive, evidenced by dark brown staining in >30% of membrane staining on tumor cells; and negative, evidenced by any lesser degree of brown staining of appreciable or non-appreciable staining on tumor cells. An H-score of membranous PD-L1 expression on the tumor cells was calculated as previously described (14).

Gene expression microarray analysis using The Cancer Genome Atlas (TCGA) dataset. We evaluated the mRNA expression levels of PD-L1 (CD274), the IFN-γ signature (19) and CD8 T effector gene signature (20) in breast invasive carcinoma tissues. The mRNA expression z-scores of genes, analyzed using the Illumina Genome Analyzer RNA Sequencing Version 2, were obtained from the TCGA breast invasive carcinoma tissue dataset through cBioPortal (http://www.cbioportal.org/) (21,22). The IFN-γ signature was calculated as the average expression level of 6 IFN-γ-related genes: indoleamine 2,3-dioxygenase 1 (IDO1), C-X-C motif chemokine ligand 10 (CXCL10), CXCL9, HLA-DRA, STAT1 and IFN-γ (19). The CD8 T effector gene signature was also calculated as the average expression level of 7 genes: CD8A, CD8B, eomesodermin (EOMES), granzyme A (GZMA), granzyme B (GZMB), IFN-γ and perforin 1 (PRF1) (20).

Statistical analysis. One-way analysis of variance followed by a Tukey's post hoc test were performed to determine the significance of the flow cytometry results. Associations between the H-score of PD-L1 and the expression of p-STAT1 and HLA class I, as well as between the number of PD-L1-positive TIICs and p-STAT1 expression in TIICs, were also assessed using the Student's t-test. Associations between p-STAT1 and HLA class I expression levels were assessed using the Chi-square test. Associations between the H-score of PD-L1 and the number of CD8-positive cells, between PD-L1 mRNA expression z-scores and the IFN-γ signature, and between PD-L1 mRNA expression z-scores and the CD8 T effector gene signature were assessed using a scatter diagram and Pearson's product moment correlation coefficient. Analyses were performed using SPSS Statistics Package version 25 (IBM Corp., Armonk, NY, USA) and a value of P<0.05 was considered to indicate a statistically significant difference.

Results
Upregulation of PD-L1 by IFN-γ in breast cancer cell lines. We have recently reported that PD-L1 expression is mainly regulated by IFN-γ via the JAK/STAT pathway, but not via the MAPK and PI3K/AKT pathways in esophageal squamous cell carcinoma cells and gastric cancer cells (14). In this study, in order to analyze PD-L1 expression in breast cancer cells, 4 breast cancer cell lines were treated with either IFN-γ (10 ng/ml), PD98059 (50 µM; MAPK inhibitor), wortmannin (1 µM; PI3K-AKT inhibitor), or lapatinib (1 µM; PI3K-AKT inhibitor), or lapatinib (1 µM) that can inhibit both the MAPK and PI3K/AKT pathways for 48 h (13). We assessed the optimal conditions of these reagents in
previous studies (13,14). As a result, PD-L1 expression was significantly upregulated in all the tested cell lines only when treated with IFN-γ (Fig. 1A).

Several previous studies have reported that IFN-γ can upregulate PD-L1 expression through the JAK/STAT and MAPK pathways in malignant tumors (23-25). Therefore, in this study, we assessed the effects of IFN-γ on both the JAK/STAT and MAPK pathways by western blot analysis and gene expression microarray analysis in the two breast cancer cell lines, BT-549 and MDA-MB-231. Western blot analysis revealed that IFN-γ treatment increased the p-STAT1 level, but not the p-ERK level in both cell lines (Fig. 1B).

**Table I. Microarray analysis in untreated and IFN-γ-treated cell lines.**

| Molecules  | BT-549 cells Untreated | IFN-γ treatment | MDA-MB-231 cells Untreated | IFN-γ treatment |
|------------|------------------------|-----------------|----------------------------|-----------------|
| PD-L1      | -11.7                  | 815.5           | 77.0                       | 110.2           |
| PD-L2      | 2.8                    | 331.0           | -28.5                      | 27.1            |
| JAK1       | 4,755.1                | 1,898.8         | 991.6                      | 725.5           |
| JAK2       | 483.1                  | 2,893.8         | 54.7                       | 132.3           |
| JAK3       | -11.4                  | -2.5            | 19.4                       | -1.3            |
| STAT1      | 7,945.8                | 17,080.4        | 2,062.7                    | 21,414.4        |
| ERK1       | 1,751.8                | 1,168.5         | 1,205.7                    | 1,126.4         |
| ERK2       | 337.5                  | 348.6           | 427.4                      | 420.7           |
| AKT1       | 3,942.4                | 3,620.0         | 3,554.2                    | 3,387.5         |
| AKT2       | -6.8                   | 45.7            | 47.9                       | 36.2            |
| AKT3       | 30.0                   | 32.3            | 38.9                       | 19.5            |
| HLA-A      | 10,130.7               | 21,337.2        | 21,600.1                   | 24,005.1        |
| HLA-B      | 4,876.9                | 18,634.7        | 16,104.7                   | 34,904.7        |
| HLA-C      | 543.5                  | 1,476.6         | 275.3                      | 999.9           |
| HLA-E      | 5,622.3                | 23,216.8        | 7,399.6                    | 20,547.7        |
| HLA-F      | 1,024.7                | 5,301.0         | 6,858.1                    | 15,147.7        |
| HLA-G      | 202.8                  | 790.5           | 1,438.5                    | 3,585.4         |
| HLA-H      | 3,053.5                | 9,808.8         | 14,781.6                   | 21,520.7        |
| HLA-DPA1   | -33.2                  | 444.8           | 4,823.8                    | 20,231.7        |
| HLA-DPB1   | -21.4                  | -25.8           | 57.4                       | 592.6           |
| HLA-DPB2   | 24.4                   | 2.0             | 1.9                        | 25.6            |
| HLA-DQA1   | -48.9                  | -21.7           | 11.9                       | 1,474.5         |
| HLA-DQA2   | 15.9                   | -4.0            | 2.1                        | 5.8             |
| HLA-DQB1   | -3.3                   | 2.4             | 86.4                       | 1,452.1         |
| HLA-DQB2   | -4.1                   | 31.8            | 26.0                       | 16.2            |
| HLA-DRA    | 114.4                  | 1,398.5         | 10,456.1                   | 37,182.3        |
| HLA-DRB1   | -19.9                  | -2.3            | 29.4                       | 78.0            |
| HLA-DRB3   | 977.7                  | 951.9           | 2,582.4                    | 8,289.0         |
| HLA-DRB4   | 129.8                  | 127.0           | 2,279.9                    | 7,899.3         |
| HLA-DRB5   | -9.3                   | -17.3           | -13.3                      | 3.4             |
| HLA-DRB6   | 25.3                   | 66.2            | 2,246.7                    | 9,342.0         |
| TAP1       | 7,741.5                | 35,696.9        | 5,333.0                    | 32,228.8        |
| TAP2       | 1,172.4                | 4,463.4         | 1,381.3                    | 4,233.3         |
| Tapasin    | 2,660.3                | 4,533.1         | 2,592.0                    | 5,062.1         |
| β2 microglobulin | 19,602.0            | 24,741.4        | 17,045.4                   | 24,486.9        |
| LMP2       | 496.3                  | 2,226.0         | 532.9                      | 4,351.8         |
| LMP7       | 1,303.3                | 4,801.6         | 1,593.7                    | 8,038.8         |
| LMP10      | 4,038.4                | 15,703.9        | 5,087.5                    | 17,694.3        |
| PA28a      | 8,930.9                | 16,073.7        | 8,037.7                    | 16,002.5        |
| PA28b      | 20,159.3               | 34,904.7        | 10,156.0                   | 25,767.6        |
| Calnexin   | 1,895.6                | 1,647.5         | 2,059.7                    | 2,221.7         |
| Calreticulin | 11,055.8              | 7,468.7         | 6,234.9                    | 9,945.8         |
we performed a gene expression microarray analysis in both cell lines, we did not perform statistical analysis for these data. Gene expression microarray analysis also revealed that PD-L1, PD-L2, HLA-A and the JAK/STAT pathway (JAK2 and STAT1) were concomitantly increased by IFN-γ in both cell lines, although there was no increase in ERK1 and ERK2 expression levels (Fig. 1C and Table I), similar to the findings of our previous studies (14,18). Taken together, IFN-γ could induce the up-regulation of PD-L1 mainly through the JAK-STAT pathway in these breast cancer cell lines.

**PD-L1 expression is upregulated in p-STAT1-positive tumors.** We then evaluated the expression levels of CD8, p-STAT1, HLA class I and PD-L1 by IHC using the specimens of 111 patients with breast cancer. The clinicopathological data are presented in Table II, and representative IHC staining for each molecule is shown in Fig. 2. Furthermore, representative IHC staining with serial sections for CD8, p-STAT1, HLA class I and PD-L1 is illustrated in Fig. 3A.

There were no significant associations or correlations observed between the number of tumor infiltrating CD8-positive T cells and the expression levels of p-STAT1 or PD-L1 (Figs. 3B and 4). Of note, in the p-STAT1-positive cases, the tumor cells exhibited significantly higher expression levels of both PD-L1 and HLA class I (P=0.003 and P=0.004, respectively) (Fig. 3C and Table III). Furthermore, in the HLA class I-positive cases, PD-L1 expression was significantly upregulated (P=0.007) (Fig. 3D). These in vivo results suggest that the PD-L1 and HLA class I expression levels are strongly related to the JAK/STAT pathway.

**PD-L1 is upregulated in p-STAT1-positive TICCs.** PD-L1 expression on TICCs was examined. Representative IHC staining images of PD-L1 and p-STAT1 expression on TICCs are presented in Fig. 5A. The number of PD-L1-positive TICCs was significantly higher in cases with p-STAT1 positively stained TICCs (Fig. 5B).

**mRNA expression of PD-L1 is significantly associated with the IFN-γ signature in breast cancer.** The gene expression data of breast invasive carcinoma tissues of 1,100 clinical cases were extracted from the TCGA database. The data revealed a significantly positive correlation between the mRNA expression levels of PD-L1 and the IFN-γ signature (Fig. 6), and between those of PD-L1 and the CD8 T effector gene signature (Fig. 7).

### Discussion

In the present study, to the best of our knowledge, we present novel and important findings relevant to anti-PD1/PD-L1 immunotherapy for breast cancer. First, the JAK/STAT pathway stimulated by IFN-γ was shown to be involved in the expression of PD-L1. Second, the expression levels of PD-L1 and HLA class I on tumor cells were simultaneously upregulated in p-STAT1-positive tumor cells, and PD-L1 expression was significantly upregulated in p-STAT1-positive TICCs. Third, there was a positive correlation between PD-L1 expression and the IFN-γ signature based on the gene expression data from the TCGA database.

### Table II. Clinical characteristics of the patients with breast cancer (n=111).

| Characteristics                        | No. of patients |
|----------------------------------------|-----------------|
| Age (years)                            |                 |
| Mean, 62.3±13.8                        |                 |
| Range, 27-93                           |                 |
| Primary tumor                          |                 |
| T1                                      | 30              |
| T2-T4                                   | 81              |
| Stage grouping                         |                 |
| II                                      | 88              |
| III                                     | 23              |
| Molecular subtypes                      |                 |
| Luminal A like                         | 42              |
| Luminal B like                         | 34              |
| Luminal-HER2                            | 7               |
| HER2                                    | 12              |
| TNBC                                    | 16              |
| ER                                      |                 |
| Positive                                | 83              |
| Negative                                | 28              |
| PgR                                     |                 |
| Positive                                | 72              |
| Negative                                | 39              |
| HER2                                    |                 |
| Positive                                | 20              |
| Negative                                | 91              |
| Ki67                                    |                 |
| <30%                                    | 68              |
| ≤30%                                    | 43              |
| Nuclear grade                           |                 |
| 1                                       | 24              |
| 2                                       | 45              |
| 3                                       | 42              |
| Histological classification            |                 |
| Invasive ductal carcinoma (total)       | 82              |
| Special (total)                        |                 |
| Mucinous carcinoma                     | 4               |
| Medullary carcinoma                    | 1               |
| Invasive lobular carcinoma             | 16              |
| Apocrine carcinoma                     | 2               |
| Spindle cell carcinoma                 | 2               |
| Invasive micropapillary carcinoma      | 1               |
| Carcinoma with neuroendocrine features | 3               |

*The grade of the tumor and stages were defined according to the UICC (TNM) classification. Luminal A like were defined as ER*, PgR >20%, HER2 and Ki67 <30%. The classification were defined according to the Japanese Breast Cancer Society (17th edition) (17). ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.*
It has been reported that conditions within the tumor microenvironment that are favorable for immunotherapy with anti-PD-1/anti-PD-L1 mAbs include greater T cell-infiltration, a significant load of neo-antigens, IFN-γ signature and expression of PD-L1 on the tumor cells (12-15), although controversy remains depending on the tumor type. These characteristics may serve as potential biomarkers for the prediction of the responsiveness to ICI. Furthermore, it is generally accepted that reliable biomarkers should be simply and easily measured in daily clinical practice. The findings of the present study indicate that p-STAT1 expression within the tumor microenvironment is a potential biomarker for immunotherapy with anti-PD-1/anti-PD-L1 mAbs in breast cancer patients. This is based on the fact that p-STAT1 expression significantly correlates with PD-L1 and HLA class I expression on tumor cells. Both factors are essential for anti-PD-1 immunotherapy (26,27), and the results of the present study indicated that both molecules were simultaneously upregulated by IFN-γ in the breast cancer microenvironment.

Other underlying mechanisms may also contribute to PD-L1 regulation in breast cancer. For instance, the involvement of the loss of phosphatase and tensin homolog (PTEN) and the ensuing activation of the PI3K pathway have been previously reported in the expression of PD-L1 (28). In the present study, our in vitro experiments and TCGA database analysis revealed...
that PD-L1 expression was mainly regulated by IFN-γ via the JAK/STAT pathway in breast cancer. 

To date, there have been several reports describing a positive correlation between the number of tumor-infiltrating CD8-positive T cells and PD-L1 expression on tumor cells (29,30). However, in the current study, we found no such correlation between them (Figs. 3B and 4). By contrast, there was a significant positive correlation between the mRNA expression levels of PD-L1 and the CD8 T effector gene signature from the TCGA dataset (Fig. 7). We speculate that the above discrepancy may be due to tumor-infiltrating CD8-positive T cells detected in the IHC analysis, including both activated effector CTLs and exhausted T cells. Furthermore, activated effector CTLs can produce IFN-γ, resulting in PD-L1 upregulation, while exhausted CD8-positive T cells do not produce IFN-γ. According to the study by Huang et al, the T cell invigoration to tumor burden ratio is associated with response to anti-PD-1 immunotherapy (31); therefore, the presence of activated effector CD8-positive T cells in the tumor microenvironment is essential for immunotherapy with anti-PD-1/anti-PD-L1 mAbs.

In addition to PD-L1 expression on tumor cells (32-34), PD-L1 expression in TICs has also been reported to play an important role in immunotherapy with anti-PD-1/anti-PD-L1

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Table III. Association between the p-STAT1 expression status and HLA class I positivity.

| p-STAT status | HLA class I | Total |
|---------------|-------------|-------|
|               | Negative    | Positive | Total |
| p-STAT1-negative | 20         | 5        | 25    |
| p-STAT1-positive  | 41         | 45       | 86    |
| Total           | 61          | 50       | 111   |

The Chi-square test was used to analyze these data (P=0.004). HLA, human leukocyte antigen; p-STAT1, phosphorylated signal transducer and activator of transcription 1.
mAbs (35,36). In the present study, we observed PD-L1 expression on TIICs (Fig. 5A) and a greater number of TIICs expressing PD-L1 on their membrane in cases with p-STAT1-positive TIICs (Fig. 5B). These observations suggest that the JAK/STAT pathway via IFN-γ may also be involved in the PD-L1 expression in TIICs. Again, p-STAT1-positivity in TIICs can possibly lead to the detection of PD-L1-expressing TIICs, which can then be reinvigorated with anti-PD-1/anti-PD-L1 mAbs, suggesting that p-STAT1-positivity within the tumor microenvironment may be a biomarker for immunotherapy with anti-PD-1/anti-PD-L1 mAbs.

Collectively, the results of the present study indicate that p-STAT1 positivity is a potential biomarker for patient selection for immunotherapy with anti-PD-1/anti-PD-L1 mAbs in breast cancer, since p-STAT1 positivity significantly reflects PD-L1 and HLA class I expressions on tumor cells as well as PD-L1 expression on TIICs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KM, YS and KK contributed to the study conception and design. KM, KS, LFK, and VK performed the cell line experiments. YN, MO, AK, SI and DI contributed to the acquisition of the patient samples. YN and TN performed and evaluated the IHC staining. YN, TT and KM analyzed the cell line and patient data. YN, KM, and HO analyzed the TCGA dataset. YN, KM, TT, DI and KK drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent forms were obtained from all the participants in this study. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation at Yamanashi University (Reference 1622) and with the Helsinki Declaration. The study was approved by the Domain Specific Review Board of the National Healthcare Group of Singapore (Reference 2015/00209).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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Figure 7. mRNA expression of PD-L1 was associated with CD8 T effector gene signature. (A) A heatmap showing mRNA expression levels of PD-L1 and the CD8 T effector gene signature in breast invasive carcinoma tissues (TCGA dataset). (B) Correlation between mRNA expression levels of PD-L1 and the CD8 T effector gene signature.
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