Clinical Significance of Expression of Immunoadjuvant Molecules (LAG-3, TIM-3, OX-40) in Neoadjuvant Chemotherapy for Breast Cancer

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Abstract. Background/Aim: Various immunosuppressive factors that inhibit the immune response to cancer are present in cancer cells and the cancer microenvironment. Co-inhibitory and co-stimulatory receptors are dynamically expressed on T-cells as immunoadjuvant molecules that regulate the state of T-cell activity. In this report we focus on immunoadjuvant molecules such as LAG-3, TIM-3, and OX-40, for which there have been few published reports. We investigated the expression of LAG-3, TIM-3 and OX-40 in tumor-infiltrating lymphocytes (TILs), and clinically verified the significance of that expression in relation to neoadjuvant thermotherapy (NAC). Patients and Methods: A total of 177 patients with resectable early-stage breast cancer were treated with NAC. Estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), Ki67, LAG-3, TIM-3 and OX-40 status were assessed by immunohistochemistry. Results: The group with low-LAG-3 expression was significantly smaller than the group with high expression in triple-negative breast cancer (TNBC) (p=0.038) and HER2-enriched breast cancer (HER2BC) (p=0.021), while the total number of pathological complete response (pCR) patients was greater (p<0.001). In TNBC and HER2BC, the pCR rate was significantly higher in the low-LAG-3 expression group (p<0.001 and p=0.02, respectively). Moreover, on multivariate analysis low-LAG-3 expression status was an independent predictor of favorable prognosis (TNBC: p=0.014, HR=8.124; HER2BC: p=0.048, HR=10.400). Conclusion: Our findings suggest that LAG-3 may become a biomarker in highly malignant breast cancers such as TNBC and HER2BC that can predict the therapeutic efficacy of NAC.

Various immunosuppressive factors that inhibit the immune response to cancer are present in cancer cells and the cancer microenvironment (1, 2). Co-inhibitory (suppression) and co-stimulatory receptors (activation) are dynamically expressed on T-cells as immunoadjuvant molecules that regulate the state of T-cell activity, and the inhibitory molecules act as immunological checkpoints. There are a number of immunological checkpoints in the progression of the immune response, and the co-inhibitory functions of receptors such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1: CD279) are considered particularly important checkpoints for regulating the autoimmune response (3, 4). Antitumor effects of immunological checkpoint inhibition therapy utilizing an anti-CTLA-4 antibody and an anti-PD-1 antibody as antitumor T-cell effectors have been discovered in many types of cancers, and these findings have dramatically changed the positioning of cancer immunotherapy in clinical settings (5-8). In breast cancer (BC) as well, it has been reported that the anti-PD-1 antibody pembrolizumab has antitumor activity toward triple-negative breast cancer (TNBC), and that the levels of expression of PD-1 and its ligand, programmed cell death-ligand-1 (PDL-1: CD274), correlate with prognosis (9-12). These findings could affect individualized therapy for breast cancer. We have previously suggested that the expression of PD-1 and PD-L1 may serve as biomarkers for predicting therapeutic efficacy in neoadjuvant chemotherapy.
(NAC) for breast cancer (13). In this report we focus on immunoadjuvant molecules such as lymphocyte activation gene-3 (LAG-3: CD223), T-cell immunoglobulin and mucin containing protein-3 (TIM-3), and orexin-40 (OX-40: CD134), for which there have been few published reports. LAG-3 works as a molecule that suppresses the proliferation and activation of T-cells, and it is also expressed by tumor-infiltrating lymphocytes (TILs), T-cells that have been impoverished by chronic infection (exhausted T cells), and by regulatory T cells (Tregs) (14-16). TIM-3 not only functions as an immunological checkpoint regulator that suppresses T-cell function synergistically with PD-1, but it also suppresses the natural immune response of myeloid-lineage cells and is responsible for important functions in cancer cell support and survival (17-19). OX-40 is an immuno-co-stimulatory molecule that is expressed by activated T-cells, is expressed by Tregs, and supports memory T-cells (20-22).

The importance of regulating and improving the immune microenvironment in cancer has been recognized because the immune microenvironment in cancer tissues affects not only the efficacy of immunotherapy, but also the efficacy and prognosis of conventional chemotherapy and other modes of anticancer therapy (23, 24). Therefore, monitoring the host's immune response to cancer in the microenvironment is believed to play a key role in predicting therapeutic efficacy and prognosis. TILs, which are considered indicators of immune response monitoring, have been reported as prognostic factors and predictors of therapeutic efficacy (25-27). In this study, we investigated the expression of LAG-3, TIM-3, and OX-40 in TILs, and clinically verified the significance of that expression in relation to NAC.

Patients and Methods

Patient background. A total of 177 patients with resectable early-stage breast cancer diagnosed as stage IIA (T1, N1, M0 or T2, N0, M0), IIB (T2, N1, M0 or T3, N0, M0) or IIIA (T1-2, N2, M0 or T3, N1-2, M0) were treated with NAC between 2007 and 2013. Tumor stage and T and N factors were stratified based on the TNM Classification of Malignant Tumours, UICC Seventh Edition (28). Breast cancer was confirmed histologically by core needle biopsy and staged by systemic imaging studies using computed tomography (CT), ultrasonography (US) and bone scintigraphy. Breast cancer was classified into subtypes according to the immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and Ki67. Based on their immunohistochemical expression, the tumors are categorized into the immuno-phenotypes of luminal A (ER+ and/or PgR+, HER2-, Ki67-low), luminal B (ER+ and/or PgR+ and HER2+) (ER+ and/or PgR+, HER2-, Ki67-high), HER2-enriched breast cancer (HER2BC) (ER-, PgR-, and HER2+) and TNBC (negative for ER, PgR and HER2). In this study, luminal A and luminal B were assumed to be hormone receptor-positive breast cancers (HRBC).

All patients received a standardized protocol of NAC consisting of four courses of FEC100 (500 mg/m² fluorouracil, 100 mg/m² epirubicin and 500 mg/m² cyclophosphamide) every 3 weeks, followed by 12 courses of paclitaxel (80 mg/m²), administered weekly (29, 30). Forty-five patients were diagnosed with HER2-positive breast cancer and trastuzumab was administered on a weekly (2 mg/kg) or tri-weekly (6 mg/kg) basis, during paclitaxel treatment (31). All patients underwent chemotherapy as outpatients. Therapeutic anti-tumour effects were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (32). Pathological complete response (pCR) was defined as the complete disappearance of the invasive compartment of the lesion with or without intraductal components, including the lymph nodes. Patients underwent mastectomy or breast-conserving surgery after NAC. All patients who underwent breast-conserving surgery were administered postoperative radiotherapy to the remnant breast. We operated for seven cases of progressive disease (PD) during the NAC enforcement when progression was confirmed. Overall survival (OS) was defined as the period from the initiation of NAC to the time of death from any cause. Disease-free interval (DFS) was defined as the interval in years from the date of the primary surgery to the first local recurrence, distant recurrences, or death from any cause. All patients were followed up by physical examination every 3 months, US every 6 months and CT and bone scintigraphy annually. The median follow-up period for the assessment of OS was 3.4 years (range=0.6-6.0 years) and 3.1 years for DFS (range=0.1-6.0 years). The design of this study is a retrospective chart review study. Written informed consent was obtained from all subjects. This research conformed to the provisions of the Declaration of Helsinki in 1995. All patients were informed of the investigational nature of this study and provided their written, informed consent. The study protocol was approved by the Ethics Committee of Osaka City University (#926).

Immunohistochemistry. All patients underwent a core needle biopsy prior to NAC, and they had undergone a curative operation involving a mastectomy or conservative surgery with axillary lymph node dissection after NAC at Osaka City University. Immunohistochemical studies were performed as previously described on core needle biopsy specimens (33). Tumor specimens were fixed in 10% formaldehyde solution, embedded in paraffin, and 4-μm-thick sections were mounted onto glass slides. Slides were deparaffinized in xylene and heated for 20 min (105˚C, 0.4 kg/m²) in an autoclave in Target Retrieval Solution. All patients underwent a core needle biopsy and trastuzumab was administered on a weekly (29, 30). Forty-five patients were diagnosed with HER2-positive breast cancer and trastuzumab was administered on a weekly (2 mg/kg) or tri-weekly (6 mg/kg) basis, during paclitaxel treatment (31). All patients underwent chemotherapy as outpatients. Therapeutic anti-tumour effects were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (32). Pathological complete response (pCR) was defined as the complete disappearance of the invasive compartment of the lesion with or without intraductal components, including the lymph nodes. Patients underwent mastectomy or breast-conserving surgery after NAC. All patients who underwent breast-conserving surgery were administered postoperative radiotherapy to the remnant breast. We operated for seven cases of progressive disease (PD) during the NAC enforcement when progression was confirmed. Overall survival (OS) was defined as the period from the initiation of NAC to the time of death from any cause. Disease-free interval (DFS) was defined as the interval in years from the date of the primary surgery to the first local recurrence, distant recurrences, or death from any cause. All patients were followed up by physical examination every 3 months, US every 6 months and CT and bone scintigraphy annually. The median follow-up period for the assessment of OS was 3.4 years (range=0.6-6.0 years) and 3.1 years for DFS (range=0.1-6.0 years). The design of this study is a retrospective chart review study. Written informed consent was obtained from all subjects. This research conformed to the provisions of the Declaration of Helsinki in 1995. All patients were informed of the investigational nature of this study and provided their written, informed consent. The study protocol was approved by the Ethics Committee of Osaka City University (#926).

Primary monoclonal antibodies directed against ER (clone 1D5, dilution 1:80; Dako, Cambridge, UK), PgR (clone PgR636, dilution 1:100; Dako), HER2 (HercepTest™; Dako), Ki67 (clone MIB-1, dilution 1:100; Dako), LAG-3 (clone 11E3, dilution 1:100, Abcam, Cambridge, UK), TIM-3 (polyclonal, dilution 1:100, Abcam), and OX-40 (clone ACT35, dilution 1:100, BD Biosciences, Franklin Lakes, NJ, USA) were used. Tissue sections were incubated with each antibody for 70 min at room temperature or overnight at 4°C, and were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig secondary antibodies [HISTOFINE (PO)™ kit; Nichirei, Tokyo, Japan]. Slides were subsequently treated with streptavidin-peroxidase reagent and were then incubated in phosphate-buffered saline-diaminobenzidine and 1% hydrogen peroxide (v/v), followed by counterstaining with Mayer’s hematoxylin. Positive and negative controls for each marker were used according to the supplier’s data sheet.
Immunohistochemical scoring. Immunohistochemical scoring was performed by two pathologists specialized in mammary gland pathology, using the blind method to confirm the objectivity and reproducibility of diagnosis. The cut-off value for ER and PgR positivity was set at ≥1% in accordance with previous studies (34). HER2 expression was scored according to the accepted grading system (0, no reactivity or membranous reactivity in less than 10% of cells; 1+, faint/barely perceptible membranous reactivity in ≥10% of cells or reactivity in only part of the cell membrane; 2+, weak to moderate complete or basolateral membranous reactivity in ≥10% of tumor cells; or 3+, strong complete or basolateral membranous reactivity in ≥10% of tumor cells). HER2 expression was considered positive if the immunostaining score was 3+, or in cases where the score was 2+ and included gene amplification determined using fluorescent in situ hybridization (FISH). For FISH analyses, each copy of the HER2 gene and its centromere 17 (CEP17) reference were counted. The interpretation followed the criteria of the ASCO/CAP guidelines for HER2 IHC classification for breast cancer; it considered positive if the HER2/CEP17 ratio was higher than 2.0 (35, 36). A Ki67-labeling index with ≥14% of tumor cells with nuclear staining was considered positive (37).

Histopathological analysis of the percentage of TILs was evaluated on a single full-face hematoxylin and eosin (HE)-stained tumor section using criteria described by Salgado et al. (38). TILs were defined as the infiltrating lymphocytes within tumor stroma and are expressed by the proportion of the field investigated (39, 40). To evaluate LAG-3, TIM-3, and OX-40 expression, the number of infiltrating T-lymphocytes stained with anti-LAG-3, anti-TIM-3 and anti-OX-40 antibody in areas surrounding cancer cells was measured under 400-times magnified microscopy in each of 5 fields of view (FOVs) selected in darkly stained areas (Figure 1A-C). Using previous reports as a reference, the median value of the average number in the 5 FOVs was determined, and that number was set as a cut-off value (9, 16, 38).

Statistical analysis. Statistical analysis was performed using the SPSS® version 19.0 statistical software package (IBM, Armonk, NY, USA). Categorical data are reported with numbers and percentages, and continuous data as a median and range. The association between LAG-3, TIM-3, OX-40, and clinicopathological variables, and the significance of different prognostic markers were analyzed using the chi-square test (or Fisher’s exact test when necessary). Association with survival was analyzed using the Kaplan–Meier plot and the log-rank test. The Cox proportional hazards model was used to compute univariable and multivariable hazards ratios (HR) for the study parameters with a 95% confidence interval (CI), and was used in a backward stepwise method for variable selection in multivariate analyses. In all of the tests, a p-value of less than 0.05 was considered statistically significant. Cut-off values for different biomarkers included in this study were chosen prior to statistical analysis.

Results

Clinicopathological responses of primary breast cancers to NAC. The BC subtypes among the 177 patients who received NAC were: TNBC in 61 (34.5%), HER2BC in 36 (20.3%), and HRBC in 80 (45.2%) patients. Treatment response was: pCR in 67 (37.9%), partial response (PR) in 84 (47.5%), stable disease (SD) in 19 (10.7%) and PD in 7 (3.9%) patients. Based on subtype, pCR was achieved in 28 (45.9%) TNBC, 18 (50.0%) HER2BC and 21 (26.3%) HRBC patients.
Expression of immunoadjuvant molecules in all breast cancer patients. Among the total number of breast cancer patients (n=177), 47 patients (26.6%; range=0 to 46%; mean=8%; median=7%; standard deviation=5%) tested positive for LAG-3 expression, 31 patients (17.5%; range=0 to 37%; mean=6%; median=6%; standard deviation=4%) tested positive for TIM-3 expression, and 32 patients (18.1%; range=0 to 38%; mean=7%; median=8%; standard deviation=4%) tested positive for OX-40 expression. When the clinicopathological features were examined, the group with low-LAG-3 expression was significantly smaller than the group with high expression in TNBC (p=0.038) and HER2BC (p=0.021), but significantly larger in HRBC (p=0.007), while the total number of pCR patients was greater (p<0.001). In the TIM-3 low-expression group, tumor diameter was significantly smaller (p=0.007) and the Ki-67 level was significantly higher (p=0.043) than in the high-expression group, and no correlation was found between intrinsic subtype and pCR rate. OX-40 expression also showed

| Parameters                              | LAG-3   | TIM-3   | OX-40   |
|-----------------------------------------|---------|---------|---------|
|                                         | Positive | Negative | Positive | Negative |
|                                         | (n=47)  | (n=130) | (n=31)  | (n=146)  |
| Age at operation ≤56                   | 18 (38.3%) | 69 (53.1%) | 14 (45.2%) | 73 (50.0%) |
|                                         | 29 (61.7%) | 61 (46.9%) | 17 (54.8%) | 73 (50.0%) |
| Age at operation >56                   | 15 (31.9%) | 57 (43.8%) | 12 (38.7%) | 60 (41.1%) |
|                                         | 32 (68.1%) | 73 (56.2%) | 19 (61.3%) | 86 (58.9%) |
| Tumor size ≤2 cm                       | 5 (10.6%) | 19 (14.6%) | 0 (0.0%)  | 24 (16.4%) |
|                                         | 42 (89.4%) | 111 (85.4%) | 31 (100.0%) | 122 (83.6%) |
| Tumor size >2 cm                       | 38 (80.9%) | 99 (76.2%) | 24 (77.4%) | 113 (77.4%) |
|                                         | 9 (19.1%) | 31 (23.8%) | 7 (22.6%)  | 33 (22.6%) |
| Lymph node status Negative              | 12 (25.5%) | 29 (22.3%) | 10 (32.3%) | 31 (21.2%) |
| Positive                                | 35 (74.5%) | 101 (77.7%) | 21 (67.7%) | 115 (78.8%) |
| Lymph node status Positive              | 7 (14.9%) | 60 (46.2%) | 18 (58.1%) | 56 (38.4%) |
| Nuclear grade 1, 2, 3                   | 38 (80.9%) | 99 (76.2%) | 24 (77.4%) | 113 (77.4%) |
|                                         | 9 (19.1%) | 31 (23.8%) | 7 (22.6%)  | 33 (22.6%) |
| Ki67 ≤14%                               | 23 (48.9%) | 51 (39.2%) | 13 (41.9%) | 90 (61.6%) |
|                                         | 24 (51.1%) | 79 (60.8%) | 18 (58.1%) | 56 (38.4%) |
| HR and HER2 status                      | 15 (31.9%) | 21 (16.2%) | 5 (16.1%)  | 31 (21.2%) |
| Non-TNBC                                | 22 (46.8%) | 39 (30.0%) | 9 (29.0%)  | 52 (35.6%) |
| Non-HER2BC                              | 25 (53.2%) | 91 (70.0%) | 22 (71.0%) | 94 (64.4%) |
| Pathological response                   | 10 (21.3%) | 70 (53.8%) | 17 (54.8%) | 63 (43.2%) |
| Positive                                | 32 (68.1%) | 109 (83.8%) | 26 (83.9%) | 115 (78.8%) |
| Pathological response                   | 37 (78.7%) | 60 (46.2%) | 14 (45.2%) | 83 (56.8%) |
| pCR                                      | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Non-pCR                                  | 40 (85.1%) | 70 (53.8%) | 10 (32.3%) | 57 (39.0%) |
| pCR                                      | 10 (21.3%) | 70 (53.8%) | 17 (54.8%) | 63 (43.2%) |
| Pathological response                   | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Positive                                | 32 (68.1%) | 109 (83.8%) | 26 (83.9%) | 115 (78.8%) |
| Pathological response                   | 37 (78.7%) | 60 (46.2%) | 14 (45.2%) | 83 (56.8%) |
| pCR                                      | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Non-pCR                                  | 40 (85.1%) | 70 (53.8%) | 10 (32.3%) | 57 (39.0%) |
| Pathological response                   | 10 (21.3%) | 70 (53.8%) | 17 (54.8%) | 63 (43.2%) |
| pCR                                      | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Non-pCR                                  | 40 (85.1%) | 70 (53.8%) | 10 (32.3%) | 57 (39.0%) |
| Pathological response                   | 32 (68.1%) | 109 (83.8%) | 26 (83.9%) | 115 (78.8%) |
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| pCR                                      | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Non-pCR                                  | 40 (85.1%) | 70 (53.8%) | 10 (32.3%) | 57 (39.0%) |
| Pathological response                   | 10 (21.3%) | 70 (53.8%) | 17 (54.8%) | 63 (43.2%) |
| pCR                                      | 7 (14.9%) | 60 (46.2%) | 21 (67.7%) | 89 (61.0%) |
| Non-pCR                                  | 40 (85.1%) | 70 (53.8%) | 10 (32.3%) | 57 (39.0%) |
| Pathological response                   | 32 (68.1%) | 109 (83.8%) | 26 (83.9%) | 115 (78.8%) |
| Pathological response                   | 37 (78.7%) | 60 (46.2%) | 14 (45.2%) | 83 (56.8%) |
no correlation between intrinsic subtype and pCR rate (Table I). In the prognostic analysis, we found a significant DFS extension in the LAG-3 low-expression group over the high-expression group \( (p=0.001, \text{log-rank}) \) (Figure 2A). We also found a significant OS extension in the LAG-3 low-expression group over the high expression group \( (p<0.001) \) (Figure 3A). There was no significant difference in DFS and OS between TIM-3 and OX-40 expression level groups (Figure 4A-D). In the univariate analysis, low-LAG-3 expression significantly contributed to extension of the disease-free survival interval \( (p=0.002, \text{HR}=3.239) \). Low-LAG-3 expression was also an independent factor for good prognosis \( (p=0.006, \text{HR}=3.047) \) in the multivariate analysis (Table II).

**LAG-3 expression in triple-negative breast cancer.** Among the 61 TNBC patients, 22 (36.1%) were in the high-LAG-3 expression group, and 39 (63.9%) were in the low-LAG-3 expression group. The pCR rate was significantly higher \( (p<0.001) \) in the low-LAG-3 expression group than in the high-LAG-3 expression group (Table III). Outcome analysis showed significantly longer DFS \( (p<0.001, \text{log-rank}) \) (Figure 2B) and OS \( (p=0.003, \text{log-rank}) \) in the low-LAG-3 expression group than that in the high-LAG-3 expression group. Low-LAG-3 expression group in patients with TNBC demonstrated significantly lower rate for the recurrence by univariate \( (p=0.003, \text{HR}=10.107) \) and multivariate analysis \( (p=0.014, \text{HR}=8.124) \) (Table II).

**LAG-3 expression in HER2 enriched breast cancer.** Among the 36 HER2BC patients, 15 (41.7%) were in the high-LAG-3 expression group, and 21 (58.3%) were in the low-LAG-3 expression group. In the clinicopathological examination,
tumor diameter was significantly smaller in the LAG-3 low-expression group than in the high-expression group ($p=0.028$), and the number of pCR patients was greater ($p=0.020$) (Table III). Outcome analysis showed significantly longer DFS ($p=0.013$, log-rank) (Figure 2C) and OS ($p=0.003$, log-rank) (Figure 3C) in the low-LAG-3 expression group than that in the high-LAG-3 expression group. On univariate analysis for recurrence, low-LAG-3 expression status was a good prognostic factor ($p=0.042$, HR=10.969). Multivariate analysis also showed that low-LAG-3 expression status was an independent good prognostic factor ($p=0.048$, HR=10.400) (Table II).

**Discussion**

Recently, cancer cells have become clear that the peripheral environment (tumor microenvironment) greatly affects cancer cells and contributes to the formation of characteristics particular to cancer (41). The immune microenvironment in tumor tissues affects not only the efficacy of immunotherapy, but also the efficacy and prognosis of chemotherapy and other modes of anticancer therapy (23, 24). Tumor antigens are present on tumor cells, and they induce an antitumor immune response in the host.
Moreover, tumors activate mechanisms to suppress antitumor immunity, particularly in the microenvironment of tumor tissue, and they utilize those mechanisms for self-proliferation (1). Furthermore, there are diverse activation and suppression signals for antitumor immunity (3). In the group of immunoadjuvant molecules that regulate T-cell activation, inhibitory molecules such as CTLA-4, PD-1, PD-L1/2, LAG-3 and TIM-3 function as immunologic checkpoints (15, 19). Furthermore, immunotherapy utilizing a blocking antibody to inhibit the signals of the immunological checkpoints has shown promising therapeutic efficacy in a clinical setting (5, 7, 8). Meanwhile, activators include OX-40, and it has been reported that administration of an anti-OX-40 agonistic antibody may enhance the antitumor immune response (42). In cancers such as malignant melanoma, renal cell carcinoma and breast cancer, a correlation has been suggested between PD-1 and PD-L1 expression levels and both the malignancy of cancer and extent of a poor prognosis (9, 11, 23). However, there have been few reports that have examined the level of expression of immunoadjuvant molecules such as LAG-3, TIM-3 and OX-40 clinically, and therefore monitoring of the tumor immune microenvironment has been conducted in NAC breast cancer patients using TILs. In this study, the LAG low-expression group had a significantly higher pCR rate than the high LAG expression group in NAC breast cancer patients, and low-LAG expression contributed to an extension of the disease-free survival interval.

LAG-3 is a molecule with a structure similar to CD4, and it appears on the cell surface when T-cells are activated (14, 16, 43). The signal transduction pathway of LAG-3 is still unclear, but it is believed to function as a molecule that not only suppresses the proliferation and activation of T-cells, but that also plays a key role as an immune checkpoint similar to PD-1 and CTLA-4 (15). Basic research has demonstrated that the antitumor immune response in mice is enhanced by inhibiting the LAG-3 signal using an anti-LAG-3 antibody while concurrently administering an anti-PD-1

Figure 4. Disease-free survival and Overall survival of the patients with all breast cancer based on the TIM-3 and OX-40 expression. There was no significant difference in disease-free survival and overall survival between TIM-3 and OX-40 expression level groups (A-D).
antibody (44). Furthermore, an anti-LAG-3 antibody was adapted for human use, and it was discovered that the use of this antibody in combination with paclitaxel in phase I and II clinical studies of breast cancer raised the response rate from 25% to 50% compared with groups treated with anticancer monotherapy (45). Paclitaxel is considered to improve the immune escape in the host by suppressing Tregs, but expression of LAG-3 has also been found in Tregs. In other words, an anti-LAG-3 antibody in combination with paclitaxel may effectively act to relieve immunosuppression by suppressing Tregs. In the NAC regimen used in our study that treats paclitaxel as a key drug, it appears that these mechanisms enable immune response monitoring via LAG-3 expression.

On the other hand, TIM-3 has galectin-9 as a ligand and suppresses the activation of effector T-cells mediated by ligand-receptor interaction (17-19). Clinical studies have reported that the level of expression of TIM-3 in renal cell carcinoma and head and neck cancer exhibits a negative correlation to prognosis (46, 47). In our study, we found no correlation between TIM-3 expression and the therapeutic efficacy of NAC. TIM-3 is a molecule that contributes to the suppression of T-cell function synergistically with PD-1, and simply activating antitumor immunity by lowering TIM-3 expression alone may not improve the tumor immune microenvironment. Moreover, OX-40 is a member of the TNF receptor superfamily, and is expressed by activated T-cells, NK cells and Tregs (20-22). It has been reported that administration of an anti-OX-40 agonistic antibody enabled rejection of fully established tumors in a mouse model (42). In our study, however, we found no correlation between OX-40 expression and therapeutic efficacy of NAC. Under the assumption that no potent antitumor effect will be obtained with anti-OX-40 agonistic antibody monotherapy, a search is now underway for a combination therapy with another drug (48). In other words, in the case of OX-40 as well, this finding indicates that a clear improvement of the tumor immune microenvironment cannot be obtained clinically through modulation of OX-40 expression alone. In addition, no correlations between expression of LAG-3, TIM-3 and OX-40 were found in this study.

In previous reports, breast cancer was not considered as a cancer that develops as the result of an immune disorder (49). Recently, however, breast cancer has come to be viewed as an immunogenic tumor, and the highly malignant subtypes TNBC and HER2BC have a high level of immune activity (50, 51). We believe that in these highly malignant breast cancers the tumor immune microenvironment can be

### Table II. Univariable and multivariable analysis with respect to disease-free survival in breast cancer subtypes.

| Parameter | Univariable analysis | Multivariable analysis |
|-----------|----------------------|------------------------|
|           | Hazard ratio | 95% CI   | p-Value | Hazard ratio | 95% CI   | p-Value |
| All breast cancers (n=177) |           |           |         |           |           |         |
| Pathological response pCR vs. non-pCR | 0.611 | 0.279-1.336 | 0.217 | 0.810 | 0.357-1.840 | 0.615 |
| LAG-3 Positive vs. Negative | 3.239 | 1.522-6.892 | 0.002 | 3.047 | 1.384-6.707 | 0.006 |
| TNBC (n=61) |           |           |         |           |           |         |
| Pathological response pCR vs. non-pCR | 0.234 | 0.050-1.084 | 0.063 | 0.602 | 0.113-3.207 | 0.552 |
| LAG-3 Positive vs. Negative | 10.107 | 2.170-47.083 | 0.003 | 8.124 | 1.520-43.420 | 0.014 |
| HER2BC (n=36) |           |           |         |           |           |         |
| Pathological response pCR vs. non-pCR | 0.464 | 0.077-2.802 | 0.402 | 0.549 | 0.089-3.401 | 0.519 |
| LAG-3 Positive vs. Negative | 10.969 | 1.093-110.131 | 0.042 | 10.400 | 1.023-105.734 | 0.048 |
| HRBC (n=80) |           |           |         |           |           |         |
| Pathological response pCR vs. non-pCR | 1.325 | 0.443-3.965 | 0.614 | 1.261 | 0.412-3.860 | 0.684 |
| LAG-3 Positive vs. Negative | 0.628 | 0.081-4.846 | 0.655 | 0.681 | 0.085-5.493 | 0.719 |

CI, Confidence interval; LAG-3, lymphocyte activation gene-3; TNBC, triple-negative breast cancer; HER2BC, human epidermal growth factor receptor 2-enriched breast cancer; HRBC, hormone receptor-positive breast cancer; pCR, pathological complete response.
Table III. Correlations between LAG-3 expression and clinicopathological parameters in 61 triple-negative, 36 HER2 enriched, and 80 luminal-type breast cancers.

| Parameters               | TNBC (n=61) | p-Value | HER2BC (n=36) | p-Value | HRBC (n=80) | p-Value |
|--------------------------|-------------|---------|--------------|---------|-------------|---------|
|                          | Positive (n=22) | Negative (n=39) | Positive (n=15) | Negative (n=21) | Positive (n=10) | Negative (n=70) |
| Age at operation ≤56    | 8 (36.4%) | 20 (51.3%) | 10 (66.7%) | 11 (72.4%) | 1 (10.0%) | 20 (28.6%) |
| ≥56                      | 14 (63.6%) | 19 (48.7%) | 6 (33.3%) | 4 (26.7%) | 5 (50.0%) | 13 (18.6%) |
| Menopause                |             |         |              |         |             |         |
| Negative                 | 6 (27.3%) | 16 (41.0%) | 4 (26.7%) | 10 (47.6%) | 5 (50.0%) | 21 (29.7%) |
| Positive                 | 16 (72.7%) | 23 (59.0%) | 11 (73.3%) | 11 (52.4%) | 5 (50.0%) | 30 (39.3%) |
| Tumor size               |             |         |              |         |             |         |
| ≤2 cm                    | 2 (9.1%) | 5 (12.8%) | 0 (0.0%) | 6 (28.6%) | 3 (30.0%) | 8 (11.4%) |
| >2 cm                    | 20 (90.9%) | 34 (87.2%) | 15 (100.0%) | 15 (71.4%) | 7 (70.0%) | 62 (80.5%) |
| Lymph node status        |             |         |              |         |             |         |
| Negative                 | 2 (9.1%) | 9 (23.1%) | 6 (40.0%) | 5 (23.8%) | 4 (40.0%) | 15 (21.4%) |
| Positive                 | 20 (90.9%) | 30 (76.9%) | 9 (60.0%) | 16 (76.2%) | 6 (60.0%) | 55 (78.6%) |
| Nuclear grade 1, 2       |             |         |              |         |             |         |
| 3                        | 16 (72.7%) | 28 (71.8%) | 13 (86.7%) | 15 (71.4%) | 9 (90.0%) | 56 (80.0%) |
| ≤14%                     | 6 (27.3%) | 11 (28.2%) | 2 (13.3%) | 6 (28.6%) | 1 (10.0%) | 14 (20.0%) |
| >14%                     | 16 (72.7%) | 27 (69.2%) | 7 (46.7%) | 13 (61.9%) | 3 (30.0%) | 38 (54.3%) |
| Ki67                      |             |         |              |         |             |         |
| ≤14%                     | 6 (27.3%) | 12 (30.8%) | 9 (60.0%) | 8 (38.1%) | 7 (70.0%) | 32 (45.7%) |
| >14%                     | 16 (72.7%) | 27 (69.2%) | 6 (40.0%) | 13 (61.9%) | 3 (30.0%) | 38 (54.3%) |
| Pathological response    |             |         |              |         |             |         |
| pCR                      | 3 (13.6%) | 25 (64.1%) | 4 (26.7%) | 14 (66.7%) | 1 (10.0%) | 20 (28.6%) |
| Non-pCR                  | 19 (86.4%) | 14 (35.9%) | 11 (73.3%) | 7 (33.3%) | 9 (90.0%) | 50 (71.4%) |

LAG-3, Lymphocyte activation gene-3; TNBC, triple-negative breast cancer; HER2BC, human epidermal growth factor receptor 2-enriched breast cancer; HRBC, hormone receptor-positive breast cancer; pCR, pathological complete response.

reliably monitored by TILs because of the high immune activity. In our study, when breast cancer was stratified by intrinsic subtype and TILs were evaluated, the groups with low-LAG expression in highly malignant breast cancers had a significantly higher pCR rate, but no significant difference was found for HRBC.

Conclusion

In TNBC and HER2BC, low LAG3 expression was shown to contribute to pCR and to have a favorable prognosis. Our findings suggest that LAG-3 may become a biomarker in highly malignant breast cancers such as TNBC and HER2BC that can predict the therapeutic efficacy of NAC.

Conflicts of Interest

None of the Authors has any conflicts of interest to disclose regarding this study.

Authors’ Contributions

All Authors were involved in the preparation of this manuscript. YA collected the data and wrote the manuscript. SK, KT, SI, WG, and TM performed the operation and designed the study. YA, SK, MS, and HT summarized the data and revised the manuscript. KH and MO provided a substantial contribution to the study design, performed the operation and revised the manuscript. All Authors read and approved the final manuscript.

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References

1. Schreiber RD, Old LJ and Smyth MJ: Cancer immunoediting: integrating immunity’s roles in cancer suppression and promotion. Science 331(6024): 1565-1570, 2011. PMID: 21436444, DOI: 10.1126/science.1203486
2. Couzin-Frankel J: Breakthrough of the year 2013. Cancer immunotherapy. Science 342(6165): 1432-1433, 2013. PMID: 24357284, DOI: 10.1126/science.342.6165.1432
3. Chen L and Flies DB: Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 13(4): 227-242, 2013. PMID: 23470321. DOI: 10.1038/nri3405
Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T and Minato N: 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 99(19): 12293-12297, 2002. PMID: 12218188. DOI: 10.1073/pnas.122461099

Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassell JC, Rutkowski P, McNeil C, Kalinka-Warzocha E, Savage KJ, Hernberg MM, Lebbe C, Charles J, Mihalciou C, Chiarion-Sileni V, Mauch C, Cognetti F, Arance A, Schmidt H, Schadendorf D, Gogas H, Lundgren-Eriksson L, Horak C, Sharkey B, Waxman IM, Atkinson V and Asciano PT: Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 372(4): 320-330, 2015. PMID: 25399552. DOI: 10.1056/NEJMoa1412082

Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Arriyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A and Szolov M: Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369(2): 122-133, 2013. PMID: 23724867. DOI: 10.1200/JCO.2013.32.0369

Topalian SL, Szolov M, McDermott DF, Kluger HM, Carvajal RD, Sharifman WH, Brahmer JR, Lawrence DP, Atkins MB, Pownderly JD, Lenjing PD, Lipson EJ, Puzanov I, Smith DC, Lever ML, Sharkey B, Waxman IM, Atkinson V and Ascierto PA: Long-term safety in patients with advanced melanoma receiving nivolumab. J Clin Oncol 32(10): 1020-1030, 2014. PMID: 24950637. DOI: 10.1200/JCO.2013.32.0369

Montaz P and Postow MA: Immunologic checkpoints in cancer therapy: focus on the programmed death-1 (PD-1) receptor pathway. Pharmacogenomics Pers Med 7: 357-365, 2014. PMID: 25484597. DOI: 10.2147/PGM.S53166

Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D and Gillanders WE: 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 139(3): 667-667, 2013. PMID: 23756627. DOI: 10.1007/s10549-013-2581-3

Sun S, Fei X, Mao Y, Wang X, Garfield DH, Huang O, Wang J, Yuan F, Sun L, Yu Q, Jin X, Wang J and Shen K: PD-L1(+)-immune cell infiltration inversely correlates with survival of operable breast cancer patients. Cancer Immunol Immunother 63(4): 395-406, 2014. PMID: 24519454. DOI: 10.1007/s00262-014-1519-x

Muenst S, Schaeerli AR, Gao F, Daster S, Trella E, Drosner RA, Muraro MG, Zajac P, Zanetti R, Gillanders WE, Weber WP and Soysal SD: Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 146(1): 15-24, 2014. PMID: 24842267. DOI: 10.1007/s10549-014-2988-5

Stagg J and Allard B: Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects. Ther Adv Med Oncol 5(3): 169-181, 2013. PMID: 23634195. DOI: 10.1177/1758884012475152

Asano Y, Kashwagi S, Goto W, Takada K, Takahashi K, Morisaki T, Fujita H, Takashima T, Tomita S, Ohsawa M, Hirakawa K and Ohira M: Prediction of treatment responses to neoadjuvant chemotherapy in triple-negative breast cancer by analysis of immune checkpoint protein expression. J Transl Med 16(1): 87, 2018. PMID: 29615063. DOI: 10.1186/s12967-018-1458-y

Triebel F, Itasukawa S, Baixera E, Roman-Roman S, Genevée C, Viegas-Pequignot E and Hercend T: LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med 171(5): 1393-1405, 1990. PMID: 1692078. DOI: 10.1084/jem.171.5.1393

Nguyen LT and Ohashi PS: Clinical blockade of PD1 and LAG3—potential mechanisms of action. Nat Rev Immunol 15(1): 45-56, 2015. PMID: 25534622. DOI: 10.1038/nri3790

Demeure CE, Wolfers J, Martin-García N, Gaulard P and Triebel F: T Lymphocytes infiltrating various tumour types express the MHC class II ligand lymphocyte activation gene-3 (LAG-3): role of LAG-3/MHC class II interactions in cell-cell contacts. Eur J Cancer 37(13): 1709-1718, 2001. PMID: 11527700. DOI: 10.1016/s0959-8490(01)00184-8

Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ and Kuchroo VK: Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 415(6871): 536-541, 2002. PMID: 11823861. DOI: 10.1038/415536a

Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, Chandwaskar R, Karman J, Su EW, Hirashima M, Bruce JN, Kane LP, Kuchroo VK and Hafler DA: Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science 318(5853): 1141-1143, 2007. PMID: 18006747. DOI: 10.1126/science.1148536

Ngio SW, Teng MW and Smyth MJ: Prospects for TIM3-Targeted Antitumor Immunotherapy. Cancer Res 71(21): 6567-6571, 2011. PMID: 22009533. DOI: 10.1158/0008-5472.CAN-11-1487

Paterson DJ, Jefferies WA, Green JR, Brandon MR, Corthesy P, Puilavee M and Williams AF: Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. Mol Immunol 24(12): 1281-1290, 1987. PMID: 2828930. DOI: 10.1016/0161-5890(87)90122-2

Vetto JT, Lun S, Morris A, Sicotte M, Davis J, Lemon M and Weinberg A: Presence of the T-cell activation marker OX-40 on tumor infiltrating lymphocytes and draining lymph node cells from patients with melanoma and head and neck cancers. Am J Surg 174(3): 258-265, 1997. PMID: 9324133. DOI: 10.1001/s0002-9610(97)00139-6

Curti BD, Kovacsovics-Bankowski M, Morris N, Walker E, Chisholm L, Floyd K, Walker J, Gonzalez I, Meeuwesen T, Fox MA, Moudgil T, Miller W, Haley D, Coffey T, Fisher B, Delanty-Miller L, Rymachynk N, Kelly T, Crocenzi T, Bernstein E, Sanborn R, Urba WJ and Weinberg AD: OX40 is a potent immune-stimulating target in late-stage cancer patients. Cancer Res 73(24): 7189-7198, 2013. PMID: 24177180. DOI: 10.1158/0008-5472.CAN-12-4174

Fridman WH, Pagès F, Sautés-Fridman C and Galon J: The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4): 298-306, 2012. PMID: 22419253. DOI: 10.1038/nrc3245

Zitvogel L, Kepp O and Kroemer G: Immune parameters affecting the efficacy of chemotherapeutic regimens. Nat Rev Clin Oncol 8(3): 151-160, 2011. PMID: 21364688. DOI: 10.1038/nrclinonc.2010.223

Liu H, Zhang T, Ye J, Li H, Huang J, Li X, Wu B, Huang X and Hou J: Tumor-infiltrating lymphocytes predict response to
chemotherapy in patients with advanced non-small cell lung cancer. Cancer Immunol Immunother 61(10): 1849-1856, 2012. PMID: 22456757. DOI: 10.1007/s00262-012-1231-7

26 Kocián P, Šedivcová M, Drgáč J, Čermá K, Hoch J, Kodet R, Bartůňková J, Špišek R and Fialová A: Tumor-infiltrating lymphocytes and dendritic cells in human colorectal cancer: their relationship to KRAS mutational status and disease recurrence. Hum Immunol 72(11): 1022-1028, 2011. PMID: 21884745. DOI: 10.1016/j.humimm.2011.07.312

27 Lee WS, Kang M, Baek JH, Lee JI and Ha SY: Clinical impact of tumor-infiltrating lymphocytes for survival in curatively resected stage IV colon cancer with isolated liver or lung metastasis. Ann Surg Oncol 20(2): 697-702, 2013. PMID: 23224827. DOI: 10.1245/s10434-012-2752-1

28 Ainbinder DJ, Esmaeli B, Groo SC, Finger PT and Brooks JP: Introduction of the 7th edition eyelid carcinoma classification system from the American Joint Committee on Cancer-International Union Against Cancer staging manual. Arch Pathol Lab Med 133(8): 1256-1261, 2009. PMID: 19653721. DOI: 10.5853/133.8.1256

29 Mauri D, Pavlidis N and Ioannidis JP: Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. J Natl Cancer Inst 97(3): 188-194, 2005. PMID: 15687361. DOI: 10.1093/jnci/dji021

30 Mieog JS, van der Hage JA and van de Velde CJ: Preoperative chemotherapy for women with operable breast cancer. Cochrane Database Syst Rev (2): CD005002, 2007. PMID: 17443564. DOI: 10.1002/14651858.CD005002.pub2

31 Buzdar AU, Valero V, Ibrahim NK, Francis D, Broglio KR, Theriault RL, Pusztai L, Green MC, Singletary SE, Hunt KK, Sahin AA, Esteva F, Symmans WF, Ewer MS, Buchholz TA and Hortobagyi GN: Neoadjuvant therapy with paclitaxel followed by fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. Clin Cancer Res 13(1): 228-233, 2007. PMID: 17200359. DOI: 10.1158/1078-0432.CCR-06-1345

32 Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D and Verweij J: New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45(2): 228-247, 2009. PMID: 19097774. DOI: 10.1016/j.ejca.2008.10.026

33 Kashiwagi S, Yashiro M, Takashima T, Aomatsu N, Kawajiri H, Kato Y, Fagarasan S, Muramatsu M, Eto T, Hioki K and Horii J: Relationship to KRAS mutational status and disease recurrence. J Immunol 180(4): 490-496, 2013. PMID: 23319435. DOI: 10.1016/j.jci.90021

34 Umemura S, Kurosumi M, Moriya T, Oyama T, Arizumi K, Yasumoto Y, Kakeji Y, Shimizu C, Fujikuma H, Kaprinos P, Kajiwara H and Akiyama F: Immunohistochemical evaluation of hormone receptors in breast cancer: a practically useful evaluation system and handling protocol. Breast Cancer 13(3): 232, 2006. PMID: 16929115. DOI: 10.2325/jbcs.13.232

35 Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical Oncology, and College of American Pathologists: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 31(31): 3997-4013, 2013. PMID: 24101045. DOI: 10.1200/JCO.2013.50.9984

36 Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical Oncology and College of American Pathologists: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. Arch Pathol Lab Med 138(2): 241-256, 2014. PMID: 24099077. DOI: 10.5858/arpa.2013-0953-SA

37 Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ and Panel members: Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22(8): 1736-1747, 2011. PMID: 21709140. DOI: 10.1093/annonc/mdr304

38 Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschens P, Pruner G, Wiebert E, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, Richardsson AL, Brook J, Criscitiello C, Bailey H, Iagnatiadis M, Floris G, Sparano J, Kos Z, Nielsen T, Rimm DL, Allison KH, Reis-Filho JS, Loibl S, Sotiriou C, Viale G, Badve S, Adams S, Willard-Gallo K, Loi S and International TILs Working Group 2014: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 26(2): 259-271, 2015. PMID: 25214542. DOI: 10.1093/annonc/mdu450

39 Ono M, Tsuda H, Shirimizu C, Yamamoto S, Shibata T, Yamamoto H, Hirata T, Yonemori K, Ando M, Tamura K, Katsuma N, Kinoshita T, Takiguchi Y, Tanzawa H and Fujiwara Y: Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. Breast Cancer Res Treat 132(3): 793-805, 2012. PMID: 21562709. DOI: 10.1007/s10549-011-1554-7

40 Mao Y, Qu Q, Zhang Y, Liu J, Chen X and Shen K: The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in triple-negative breast cancer. Breast Cancer Res Treat 132(3): 793-805, 2012. PMID: 21562709. DOI: 10.1007/s10549-011-1554-7

41 Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell 144(5): 646-674, 2011. PMID: 21376230. DOI: 10.1016/j.cell.2011.02.013

42 Weinberg AD, Rivera MM, Prell R, Morris A, Ramstad T, Vetto JT, Urba WJ, Alvord G, Bunce C and Shields J: Engagement of the OX-40 receptor in vivo enhances antitumor immunity. J Immunol 164(4): 2160-2169, 2000. PMID: 10657670. DOI: 10.4049/jimmunol.164.4.2160

43 Baxieras E, Huard B, Miossec C, Jitsukawa S, Martin M, Herceg T, Auffray C, Tribel F and Pierrat-Tonneau D: Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. J Exp Med 176(2): 327-337, 1992. PMID: 1380059. DOI: 10.1084/jem.176.2.327

44 Okazaki T, Okazaki IM, Wang J, Sugiuara D, Nakaki F, Yoshida T, Kato Y, Fagarasan S, Muramatsu M, Eto T, Hioki K and Honjo T:
PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. J Exp Med 208(2): 395-407, 2011. PMID: 21300912. DOI: 10.1084/jem.20100466

45 Brignone C, Gutierrez M, Mefti F, Brain E, Jarcau R, Cvitkovic F, Bousseta N, Medioni J, Gligorov J, Grygar C, Marcu M and Triebel F: First-line chemoimmunotherapy in metastatic breast carcinoma: combination of paclitaxel and IMP321 (LAG-3Ig) enhances immune responses and antitumor activity. J Transl Med 8: 71, 2010. PMID: 20653948. DOI: 10.1186/1479-5876-8-71

46 Yuan J, Jiang B, Zhao H and Huang Q: Prognostic implication of TIM-3 in clear cell renal cell carcinoma. Neoplasma 61(1): 35-40, 2014. PMID: 24195506.

47 Cao Y, Zhou X, Huang X, Li Q, Gao L, Jiang L, Huang M and Zhou J: Tim-3 expression in cervical cancer promotes tumor metastasis. PLoS One 8(1): e53834, 2013. PMID: 23335978. DOI: 10.1371/journal.pone.0053834

48 Hirschhorn-Cymerman D, Budhu S, Kitano S, Liu C, Zhao F, Zhong H, Lesokhin AM, Avogadri-Connors F, Yuan J, Li Y, Houghton AN, Merghoub T and Wolchok JD: Induction of tumoricidal function in CD4+ T cells is associated with concomitant memory and terminally differentiated phenotype. J Exp Med 209(11): 2113-2126, 2012. PMID: 23008334. DOI: 10.1084/jem.20120532

49 Rosenberg SA, Yang JC and Restifo NP: Cancer immunotherapy: moving beyond current vaccines. Nat Med 10(9): 909-915, 2004. PMID: 15340416. DOI: 10.1038/nm1100

50 Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, Martino S, Wang M, Jones VE, Saphner TJ, Wolff AC, Wood WC, Davidson NE, Sledge GW, Sparano JA and Badve SS: Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol 32(27): 2959-2966, 2014. PMID: 25071121. DOI: 10.1200/JCO.2013.55.0491

51 Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, Pfützner BM, Salat C, Losi S, Schmitt WD, Schmehl C, Fisch K, Dab-Esfahani S, Mehta K, Sotiriou C, Wienert S, Klare P, André F, Klauschen F, Blohmer JU, Krappmann K, Schmidt M, Tesch H, Kümmel S, Sinn P, Jackisch C, Dietel M, Reimer T, Untch M and Loibl S: Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol 33(9): 983-991, 2015. PMID: 25534375. DOI: 10.1200/JCO.2014.58.1967

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