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Lymphocyte-activating gene-3 expression is associated with tumor-infiltrating lymphocyte levels in HER2-positive breast cancers

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Abstract

Lymphocyte-activating gene-3 (LAG-3, CD223) is the third inhibitory receptor targeted for immunotherapy. Several clinical trials investigating the use of interventions targeting LAG-3 are underway. The exact signaling mechanism downstream of LAG-3 is largely unknown, especially in breast cancer. The prognostic significance of tumor-infiltrating lymphocytes (TILs) in breast cancer has been previously determined.

Among 167 human epidermal growth factor receptor 2 (HER2)-positive breast cancer patients, 90 and 78 patients were positive and negative for the hormone receptor, respectively. LAG-3 mRNA and protein expression levels in TILs were evaluated by quantitative real-time polymerase chain reaction and immunohistochemistry, respectively, among 12 and 167 HER2-positive breast cancer samples, respectively.

High expression of LAG-3 in TILs was significantly correlated with high levels of TILs ($P = .003$) and an abundance of tertiary lymphoid structures around invasive components ($P = .014$). In addition, high expression of LAG3 was significantly associated with positivity for programmed death-ligand 1 (PD-L1) in tumor cells, a high immunostaining score of PD-L1 in TILs, and a high total immunostaining score for PD-L1 in tumor cells and TILs (all, $P < .001$). High expression levels of LAG-3 mRNA were associated with high levels of TILs ($P = .091$).

LAG-3 protein expression was not a prognostic factor in HER2-positive breast cancers, and LAG-3 expression in TILs was significantly associated with the levels of TILs in HER2-positive breast cancer, although it was not a prognostic factor.

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, HR = hormone receptor, LAG-3 = lymphocyte-activating gene-3, ORR = objective response rate, PD-L1 = PD-ligand 1, PR = progesterone receptor, SISH = silver *in situ* hybridization, TIL = tumor-infiltrating lymphocyte, TLS = tertiary lymphoid structure.

Keywords: breast cancer, human epidermal growth factor receptor 2, lymphocyte-activating gene-3, tumor-infiltrating lymphocytes

1. Introduction

Immune checkpoint inhibitors, especially those targeting the cytotoxic T lymphocyte-associated antigen 4 and programmed death 1/PD-ligand 1 (PD-L1) axis, show impressive results in several tumor types. However, most patients do not respond to this treatment.[1–3] Therefore, many studies have focused on combination therapies and the identification of alternative inhibitory receptors. Many clinical trials have been conducted to determine the optimal interventions for combination with immunotherapeutics, such as antagonistic monoclonal antibodies targeting different inhibitory receptors and other classic modalities encompassing chemotherapy, radiation therapy, and/

Editor: Kamalika Mojumdar.

This study was supported by a Biomedical Research Institute Grant (2017-B033) from Pusan National University Hospital (Seokwon Lee).

The authors have no conflicts of interests to disclose.

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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How to cite this article: Lee S, Kim JY, Lee SJ, Kwon SW, Jung HJ, Jung SJ, Kim KB, Choi KU, Lee CH, Huh GY, Kim A. Lymphocyte-activating gene-3 expression is associated with tumor-infiltrating lymphocyte levels in HER2-positive breast cancers. Medicine 2021;100:50(e28057).

Received: 6 June 2021 / Received in final form: 11 October 2021 / Accepted: 8 November 2021

http://dx.doi.org/10.1097/MD.0000000000028057

or cancer vaccination. Lymphocyte-activating gene-3 (LAG-3) is the third inhibitory receptor clinically targeted.[4] Historical clinical trial data revealed a 50% objective response rate (ORR) with a combinatorial therapy of IMP321, an LAG-3 modulating agent, and first-line paclitaxel treatment, compared with 25% ORR with paclitaxel monotherapy in metastatic breast cancers.[5] Moreover, clinical trials specifically targeting breast cancer are underway.

To date, agents that block the programmed death 1/PD-L1 axis, especially pembrolizumab and atezolizumab, are the most extensively studied checkpoint inhibitors in breast cancers.[6,7] The PANACEA trial (NCT02129556; KEYNOTE-014) demonstrated that combinatorial treatment with pembrolizumab and trastuzumab is effective and offers long-term clinical benefits in trastuzumab-resistant, PD-L1-positive, human epidermal growth factor receptor 2 (HER2)-positive breast cancers.[8] We previously reported that PD-L1-positive tumor cells are present in 48.5% of HER2-positive breast cancers, and tumor-infiltrating lymphocytes (TILs) in 31.7% of patients with HER2-positive breast cancer exhibit high levels of PD-L1 expression.[9] In addition, co-blocking of the LAG-3 and PD-1 pathways produces a synergistic effect that enhances the anti-tumor CD8+ T cell response in animal models.[10] Importantly, the simultaneous blockade of PD-1 and LAG-3 resulted in objective responses in 11% of melanoma patients who exhibited disease progression after anti-PD(L)1 monotherapy (NCT01968109).[11,12] Although preliminary data have already suggested the clinical efficacy of LAG-3-blocking agents, little is currently known regarding LAG-3 in cancers, including its mechanism of action.

The immune microenvironment affects tumor progression and response to treatment.[13] TILs have a prognostic impact in various tumors, including HER2-positive breast cancers.[14,15] Tertiary lymphoid structures (TLSs) control the tumor invasion and metastasis and TLS density has prognostic impact on various cancers including breast cancer.[16] In a previous study, we showed that the expression of PD-L1 in tumor cells and TILs was significantly associated with the level of TILs in HER2-positive breast cancers.[9] Although LAG3 expression in breast cancers has been investigated in several studies, limited information is available regarding the relationship between LAG3 expression and TIL levels in HER2-positive breast cancers.

In this study, we evaluated LAG3 expression in HER2-positive breast cancers and correlated them with various clinicopathological factors, including TIL levels, using immunohistochemistry and quantitative real-time polymerase chain reaction. In addition, the association between LAG-3 and PD-L1 expression was analyzed. Furthermore, we assessed the prognostic significance of LAG3 expression in HER2-positive breast cancers.

2. Methods

2.1. Patients and tissue specimens

We evaluated 167 patients with HER2-positive breast cancer who underwent surgery for primary breast cancer between 2011 and 2013 at Pusan National University Hospital. Formalin-fixed paraffin-embedded tissue blocks were available for all the patients. All patients were chemotherapy- and radiotherapy-naïve. After surgery, all the patients received adjuvant treatment. They were treated with 4 cycles of anthracycline and cyclophosphamide (AC) and trastuzumab (1 year) as standard treatments. Paclitaxel or docetaxel was administered between 4 cycles of AC and 1 year of trastuzumab, based on risk stratification, depending on the following: histological grade, tumor size, Ki-67 proliferation index, lymph node metastasis, age, family history of breast cancer, and general conditions. During treatment, 14 patients discontinued trastuzumab, 3 showed cardiac toxicity, 4 died, and 11 had recurrence or metastasis. Clinical information was collected from electronic medical records, and pathology data were obtained from pathology reports. According to reviewing medical records, all patients had no special genetic background. The biospecimens were provided by the Biobank of Pusan National University Hospital, a member of the National Biobank of Korea. All samples from the National Biobank of Korea were obtained with informed consent in accordance with institutional review board-approved protocols.

2.2. Histological evaluation

All available hematoxylin and eosin-stained slides were reviewed. TIL levels were evaluated in accordance with the recommendations of the International TILs Working Group.[17] TIL levels were assessed as percentages in increments of 10%. If the level of TILs was less than 10%, 1% or 5% criteria were used. TLS is a lymph node-like structure characterized by the ectopic aggregation of lymphoid cells with specialized high endothelial venules. Lymphoid aggregation-showing vessels with high endothelial venule features (plump and cuboidal endothelial cells) were considered TLSs. The ducts affected by DCIS that had TLSs surrounding them were evaluated, and TLSs as a percentage of the total circumference of the invasive front were assessed. Other histopathological factors, including nuclear grade, histologic grade, necrosis, lymphovascular invasion, and lymph node metastasis were also evaluated. Nuclear and histological grades were based on the modified Bloom-Richardson classification.

Patients were categorized into 3 groups ($\leq$10%, 20%–60%, and $>$ 60%) or 2 groups ($\leq$60% and $>$60%) based on the level of TILs, as appropriate for statistical analysis. TLSs were stratified into 2 subgroups based on their levels (low and high, according to the mean value).

2.3. Generation of tissue microarray blocks and immunohistochemistry

The tissue microarray blocks comprised tissue cores 2 mm in diameter. To minimize tissue loss and the effects of tumor heterogeneity, all samples were arrayed in duplicate.

At the time of diagnosis, immunohistochemistry was performed to check the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 using Benchmark XT (Ventana Medial System, Tuscon, AZ, USA). For deparaffinization, EZ prep was used with an auto-staining device on 3 μm paraffin sections. Cell conditioner 1 (CC1; pH 8.4 buffer) or CC2 (pH 6.0 buffer) were used for antigen retrieval. Optimally formulated antibodies targeting ER (SP1, predilute, Ventana Medical Systems), PR (1E2, prediluted, Ventana Medical Systems), and HER-2/neu (4B5, predilute, Ventana Medical Systems) were utilized. Samples were considered positive for ER and PR in immunohistochemistry if the tumor nuclei showed positive staining in more than 1% of the total tumor nuclei.

Tumors with ER or PR positivity were classified as hormone receptor (HR)-positive. HER2 positivity was defined as an immunostaining score of 3 or by gene amplification confirmed by silver in situ hybridization (SISH). Tumors were categorized into 2 subtypes based on HR (ER and PR) expression status: HR +/HER2+ and HR-/HER2+.

In cases of equivocal immunohistochemistry with a score of 2, SISH was performed for HER2. A HER2/CEP17 chromosome dual-probe (Ventana Medical Systems) was used for SISH.

Immunohistochemistry was performed to check the expression of LAG-3 in tissue microarray sections using BOND-MAX (Leica Biosystems, Germany). The Bond Dewax Solution was used for deparaffinization and the Bond Epitope Retrieval Solution for antigen retrieval. Immunohistochemistry was performed using an anti-LAG-3 antibody (EPR4392, 1;1000, Abcam). We used human tonsils as the positive control for LAG-3, and the positive control was used for each section of the tissue microarray. The expression of LAG-3 in the TILs was evaluated. Both intensity and percentage of positivity were considered collectively (Fig.1). The intensity of positivity ranged from 0 (no expression) to 3 (strong positivity). The predominant staining intensity was used if the staining intensity showed heterogeneity. The immunostaining score was calculated by multiplying the percentage of positive cells by the staining intensity. LAG-3 expression was stratified into 2 categories (low and high) based on the median value of the immunostaining score. PD-L1 (1:200, E1L3N, Cell Signaling Technology, Canvers, MA, USA) expression was independently evaluated in tumor cells and TILs, and the details are described in our previous study.[9]

2.4. Quantitative real-time polymerase chain reaction for checking the expression of LAG-3 mRNA

Total RNA was isolated from 12 HER2-positive breast cancer samples using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A ProtoScript First Strand cDNA Synthesis Kit (New England Biolab Inc., MA, USA) was used to synthesize cDNA from total RNA. Luna Universal qPCR Master Mix (New England Biolab Inc., MA, USA) and primers were used to determine RNA expression. Quantitative PCR reactions were performed on an ECO Real-Time PCR system and analyzed using ECO software (PCRmax, Beacon Road, Staffordshire, UK). Each experiment was performed in triplicate. The comparative CT method (ΔΔCT method) was used to determine the relative expression of LAG-3 mRNA. The mRNA expression of LAG-3 was normalized to that of β-actin. LAG-3 mRNA expression was stratified into 2 subgroups (low and high) based on the mean fold change.

2.5. Statistical analysis

All statistical analyses were performed using the SPSS statistical software (version 11; IBM, Armonk, NY). The Chi-Squared test, Fisher exact test, and Kaplan–Meier survival analyses were employed as appropriate. Differences with *P* values of less than .05, were considered significant.

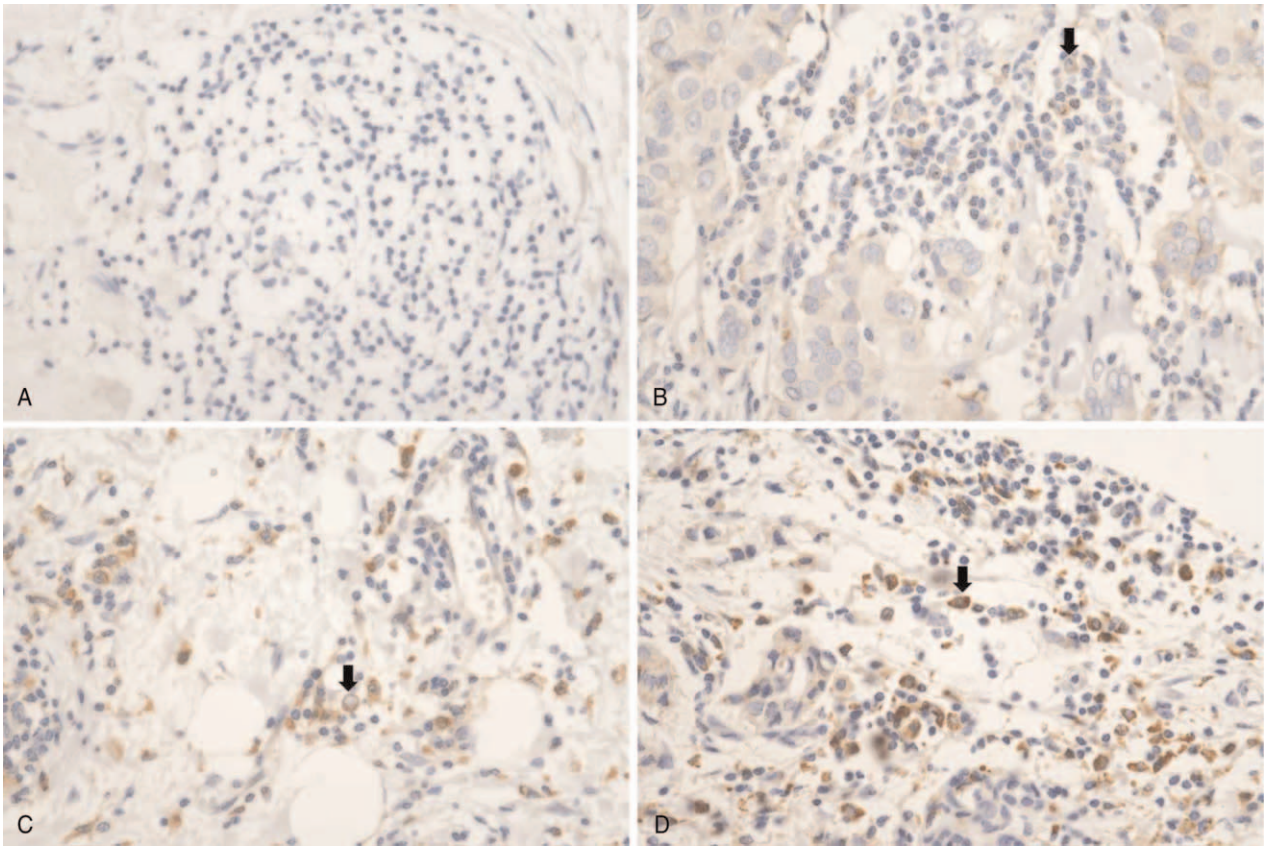

Figure 1. LAG-3 expression in TILs evaluated using immunohistochemistry. Positive staining shown in brown color and representative cells indicated by arrow. (A) No expression, (B) intensity of 1, (C) intensity of 2, and (D) intensity of 3.

Table 1

Correlation between lymphocyte-activating gene-3 expression evaluated using immunohistochemistry and variable clinicopathological parameters.

	LAG-3 expression in tumor infiltrating lymphocytes (TILs)		
	Low	High	*P* value
Age (yr)	52.21 ± 9.487	52.99 ± 10.809	.261
Size (cm)	3.138 ± 1.979	3.291 ± 1.533	.715
Side			1.000
Right	48 (55.8)	45 (55.6)	
Left	38 (44.2)	36 (44.4)	
Histological grade			.112
Low (grade 1 and 2)	37 (43.0)	25 (30.9)	
High (grade 3)	49 (57.0)	56 (69.1)	
Nuclear grade			.688
Low (grade 1 and 2)	16 (18.6)	13 (16.0)	
High (grade 3)	70 (81.4)	68 (84.0)	
Necrosis			1.000
Absent	29 (33.7)	28 (34.6)	
Present	57 (66.3)	53 (65.4)	
Microcalcification			.537
Absent	39 (45.3)	41 (50.6)	
Present	47 (54.7)	40 (49.4)	
Lymphovascular invasion			.756
Absent	45 (52.3)	45 (55.6)	
Present	41 (47.7)	36 (44.4)	
Lymph node metastasis			1.000
Absent	40 (46.5)	38 (46.9)	
Present	46 (53.5)	43 (53.1)	
Stage			.285
Early (stage I & II)	68 (79.1)	58 (71.6)	
Advanced (stage III & IV)	18 (20.9)	23 (28.4)	
Hormone receptor expression			.217
Positive	50 (58.1)	39 (48.1)	
Negative	36 (41.9)	42 (51.9)	
TIL level			**.003**
≤10%	40 (46.5)	21 (25.9)	
20%–60%	35 (40.7)	39 (48.1)	
>60%	11 (12.8)	21 (25.9)	
Tertiary lymphoid structure around invasive component			**.014**
Low	64 (74.4)	45 (55.6)	
High	22 (25.6)	36 (44.4)	
Tertiary lymphoid structure around DCIS			.875
Low	52 (60.5)	50 (61.7)	
High	34 (39.5)	31 (38.3)	

3. Results

3.1. LAG-3 expression on TILs in HER2-positive breast cancers

Among the 167 patients with HER2-positive breast cancer, 86 (51.5%) had low expression of LAG-3 on TILs, and 81 (48.5%) had high expression. The association between LAG-3 expression and various clinicopathological factors is shown in Table 1. A high level of LAG-3 expression on TILs was significantly correlated with a high TIL level ($P = .003$) and abundance of TLSs around the invasive components ($P = .014$) (Fig. 2).

3.2. LAG-3 and PD-L1 expression in HER2-positive breast cancers

Table 2 shows the correlation between LAG-3 and PD-L1 expression based on the immunohistochemistry results. High LAG-3 expression in TILs was significantly associated with the following: PD-L1 positivity of tumor cells, high immunostaining score for PD-L1 on TILs, and high total immunostaining score for PD-L1 on tumor cells and TILs ($P < .001$, all).

3.3. LAG-3 mRNA expression in HER2-positive breast cancers

The fold difference in LAG-3 mRNA expression ranged from 0.456 to 12.625 (2.280 ± 3.439). Nine samples (75.0%) were categorized as having low expression of LAG-3 mRNA, and the other 3 samples (25.0%) were categorized as having high expression of LAG-3 mRNA based on the mean fold change (mean value 2.280). The *t* test revealed that the fold change in LAG-3 mRNA expression was different between the low and high TIL groups (0.8819 ± 0.3923 vs 4.2390 ± 4.9066, respectively, $P = .096$), although the difference was not significant. High levels of LAG-3 mRNA expression were associated with high TIL levels, although this association was not significant ($P = .091$). In

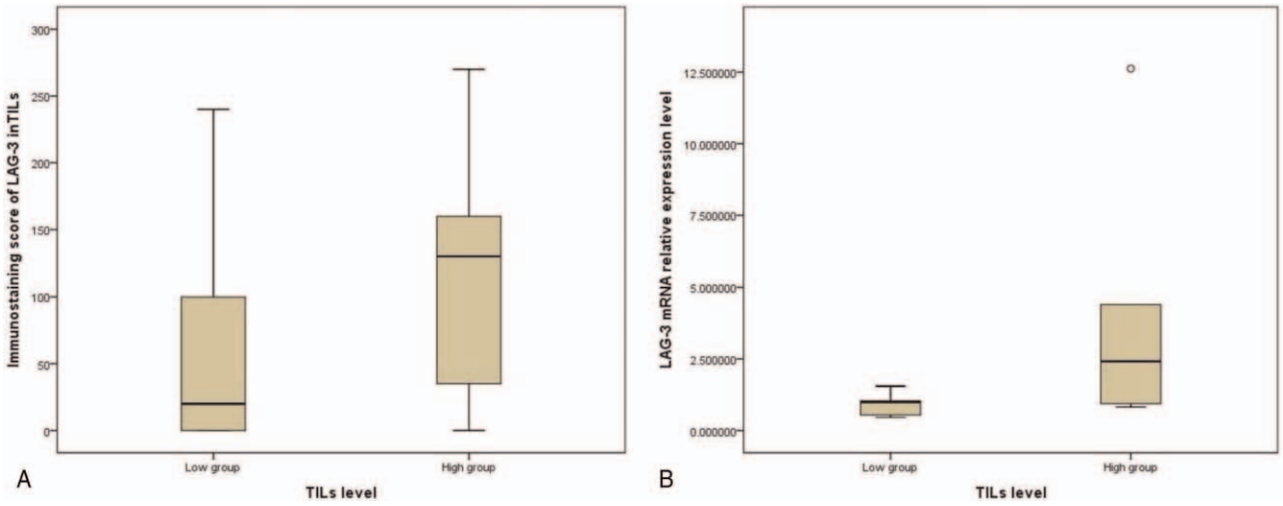

Figure 2. LAG-3 expression based on the levels of tumor infiltrating lymphocytes (TILs). (A) Immunostaining score of LAG-3 in TILs based on the TIL levels. (B) Relative LAG-3 mRNA expression based on TIL levels.

addition, high levels of LAG-3 mRNA expression were significantly correlated with the abundance of TLSs around the invasive components and DCIS components ($P = .005$ and $P = .045$, respectively) (Table 3).

3.4. Prognostic significance of LAG-3 expression in HER2-positive breast cancers

The median follow-up time was 1942 days (range, 82–2596 days). During the follow-up period, 16 patients died from the disease. In addition, 7 patients (4.2%) experienced local recurrence (4.2%) and 23 (13.8%) experienced distant metastasis. However, LAG-3 expression at the protein and mRNA levels did not exhibit prognostic significance in patients with HER2-positive breast cancer.

4. Discussion

LAG-3 was discovered by Triebel et al as a novel transmembrane protein in activated human NK and T-cell lines in 1990.[18] *LAG-3* and *CD4* are evolutionarily similar, but the proteins encoded by these 2 genes share only ~20% sequence homology. LAG-3 can bind major histocompatibility complex class II, as the extracellular folding patterns are the same as those of CD4. In addition, LAG-3 exhibits a greater affinity for major histocompatibility complex class II than CD4.[19] The mechanism by which LAG-3 transduces downstream signals has not yet been determined. However, it is known that LAG-3 has a unique cytoplasmic domain that is not shared by other inhibitory receptors, indicating that LAG-3 has a distinct mode of action.[4]

LAG-3 is an activation marker for CD4+ and CD8+ T cells in physiological settings. In mice, LAG-3 was first detected 24 hours after stimulation *in vitro*, and its expression peaked at 48 hours and declined by day 8. Constant T cell activation leads to sustained expression of LAG-3 on T cells that frequently co-express additional inhibitory receptors, resulting in a state of T cell dysfunction.[4,20] Thymus-derived regulatory T cells, plasmacytoid dendritic cells, and a small percentage of γδT cells constitutively express LAG-3.[21,22]

Antitumor defense occurs not only in secondary lymphoid organs like lymph nodes but also directly at the tumor site within organized cellular aggregates, which are called tertiary lymphoid structures (TLSs). TLSs provides privileged sites for presentation of tumor antigens to T cells by dendritic cells. Also, they function as specialized site for activation, proliferation and differentiation of T and B cells leading to production of effector memory T helper cells and effector memory cytotoxic cells, memory B cells and antibody producing plasma cells.[16,23] In lung tumors, high density of mature dendritic cells in TLSs correlated with high level of CD8+ T cell infiltration in tumor.[24] This study showed the

Table 2

Correlation between lymphocyte-activating gene-3 and programmed death-ligand 1 expression evaluated using immunohistochemistry.

	LAG-3 expression in tumor infiltrating lymphocytes (TILs)		
	Low	High	*P* value
PD-L1 expression on tumor cells based on Allred score			<.001
Absent	63 (73.3)	23 (28.4)	
Present	23 (26.7)	58 (71.6)	
PD-L1 expression on TILs			<.001
Low	76 (88.4)	44 (54.3)	
High	10 (11.6)	37 (45.7)	
Total PD-L1 expression on tumor cells and TILs			<.001
Low	74 (86.0)	40 (49.4)	
High	12 (14.0)	41 (50.6)	

Table 3

Correlation between lymphocyte-activating gene-3 mRNA expression and variable clinicopathological parameters in 12 human epidermal growth factor receptor 2-positive breast cancer patients.

	LAG-3 mRNA expression		*P* value
	Low	High	
Age (yr)	60.22 ± 11.819	51.67 ± 4.041	.092
Size (cm)	4.367 ± 2.375	4.000 ± 1.113	.729
Side			1.000
Right	8 (88.9)	3 (100.0)	
Left	1 (11.1)	0 (0.0)	
Histological grade			1.000
Low (grade 1 and 2)	3 (33.3)	1 (33.3)	
High (grade 3)	6 (66.7)	2 (66.7)	
Nuclear grade			.509
Low (grade 1 and 2)	3 (33.3)	0 (0.0)	
High (grade 3)	6 (66.7)	3 (100.0)	
Necrosis			1.000
Absent	2 (22.2)	1 (33.3)	
Present	7 (77.8)	2 (66.7)	
Microcalcification			1.000
Absent	4 (44.4)	1 (33.3)	
Present	5 (55.6)	2 (66.7)	
Lymphovascular invasion			.491
Absent	2 (22.2)	2 (66.7)	
Present	7 (77.8)	1 (33.3)	
Lymph node metastasis			1.000
Absent	2 (22.2)	1 (33.3)	
Present	7 (77.8)	2 (66.7)	
Stage			1.000
Early (stage I & II)	4 (44.4)	2 (66.7)	
Advanced (stage III & IV)	5 (55.6)	1 (33.3)	
Hormone receptor expression			1.000
Positive	4 (44.4)	2 (66.7)	
Negative	5 (55.6)	2 (33.3)	
Tumor infiltrating lymphocytes			.091
≤10%	5 (55.6)	0 (0.0)	
20%–60%	2 (22.2)	0 (0.0)	
>60%	2 (22.2)	3 (100.0)	
Tertiary lymphoid structure around invasive component			**.005**
Low	9 (100.0)	0 (0.0)	
High	0 (0.0)	3 (100.0)	
Tertiary lymphoid structure around DCIS			**.045**
Low	9 (100.0)	1 (33.3)	
High	0 (0.0)	2 (66.7)	

association of LAG3 high expression on TILs with high TIL level and abundance of TLSs around invasive component, and association of high mRNA LAG3 level with abundance of TLSs around the invasive component and DCIS component. All these results are in conjunction with that LAG3 is an activation marker of T cells and TLSs are the specialized sites for the antitumor defense generating effector cells.

LAG-3 expression on TILs may be employed as a predictive biomarker for clinical benefits by using an antagonistic monoclonal antibody against LAG-3. According to Ascierto et al, the ORR is higher in advanced melanoma patients with LAG-3 expression in TILs than in those with LAG-3 negative TILs, irrespective of PD-L1 status.[12,25] We showed that 86 out of 167 (51.5%) patients with HER2-positive breast cancer had high expression of LAG-3 on TILs, although the cut-off value was different. This suggests that more than half of the patients with HER2-positive breast cancer could benefit from treatment with an anti-LAG-3 agent. In addition, several bispecific antibodies that target both PD-l and LAG-3 or PD-L1 and LAG-3—to block both inhibitory pathways to synergize their effects, have been developed.[25–27] With respect to tumor positivity for PD-L1, more than 58 patients (34.7% of cohort) with HER2-positive breast cancers can consider using bispecific antibodies according to this study (Table 2).

Limited data are available regarding LAG-3 expression in breast cancer patients. Du et al showed that LAG-3 was up-regulated in breast cancer and LAG3 mRNA expression had significantly better association with relapse free survival.[28] Also, high LAG-3 mRNA expression was significantly related with longer overall survival in TNBC. Bottai et al showed that PD-1 and LAG-3 were concurrently expressed on TILs in approximately 15% of patients with triple-negative breast cancer, and the expression of both checkpoint receptors was positively associated with the presence of TILs, although it was not a significant prognostic factor.[29] Another study investigated LAG-3 expression in triple negative breast cancers. Wang et al reported that LAG-3 expression is significantly correlated with the pathological complete response in pre-neoadjuvant chemotherapy specimens, and that high expression of LAG-3 had a

significant effect on nodal status in postneoadjuvant chemotherapy specimens in triple-negative breast cancers. In addition, high expression of LAG-3 in residual tissues has been correlated with poor prognosis.[30] Burugu et al evaluated the expression of LAG-3 in TILs in breast cancer in a large cohort of 4322 breast cancer patients. LAG-3-positive intraepithelial TILs were significantly associated with HER2 and basal-like breast cancer subtypes, and significantly improved breast cancer-specific survival in patients with ER-negative breast cancer, HER2, and basal-like subtypes.[31] We showed that LAG-3 expression is not a prognostic factor. Compared with the results of Burugu et al , the total follow-up period was not sufficient to draw any conclusive results. To the best of our knowledge, this is the first study on LAG-3 expression and TILs in patients with HER2-positive breast cancer.

A better understanding of LAG-3 is critical for the clinical application of LAG-3 immunotherapy. Further studies are necessary to delineate the expression and mechanism of action of LAG-3.

Acknowledgments

The biospecimens and data used in this study were provided by the Biobank of Pusan National University Hospital, a member of the Korea Biobank Network.

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