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Differential expression of novel immune checkpoint receptors on tumor-infiltrating lymphocytes in patients with locally advanced breast cancer after neoadjuvant chemotherapy

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Immune checkpoint receptors (ICRs) were recently found to modulate the anti-tumoral immune response. This study aimed to determine the clinical and pathological associations of ICRs expression on tumor-infiltrating lymphocytes (TILs) in patients with locally advanced breast cancer (LABC) treated with neoadjuvant chemotherapy (NAC). Expressions of ICRs including PD-1, LAG-3, TIM-3, TIGIT, and CTLA-4 on $CD8^+$ T lymphocytes and Natural Killer (NK) cells on TILs were analyzed by flow cytometry. Patients <50 years were more likely to express CTLA-4 on $CD8^+$ T lymphocytes compared to those ≥50 years (p=0.004). In addition, patients with ypT3-4 tumors were more likely to have increased LAG-3 expression on CD16-$CD56^{bright}$ NK cells (p=0.042) and PD-1 (p=0.014) and CTLA-4 (p=0.018) expressions on $CD8^+$ T cells in regard to those with ypT1-T2, respectively. Contrarily, PD-1 expression on CD16-$CD56^{bright}$ NK cells was found to be decreased in patients with ypN+ compared to those with ypN– (p=0.022). Furthermore, patients with HER2+ tumors were more likely to have increased TIM-3 expression on CD8+ T cells (p=0.043), whereas patients with a better response to NAC were more likely to express TIGIT on $CD8^+$ T (p=0.014) and CD16-$CD56^{bright}$ NK cells (p=0.003), respectively. The new generation ICRs, TIM-3, LAG-3, and TIGIT are highly expressed in LABC following NAC in patients with poor prognostic factors. Therefore, new evolving therapies using inhibitory mAbs directed to TIM-3, LAG-3, and TIGIT could be also be considered in locally advanced breast cancers expressing these ICRs.

Key words: locally advanced breast cancer, PD-1, TIGIT, CTLA-4, LAG-3, TIM-3

Immune checkpoint receptors (ICRs) were recently found to modulate the anti-tumoral immune response [1]. The effectiveness of targeted immunotherapy by inhibiting immune checkpoints has been investigated not only in lung cancer and malignant melanoma but also in breast cancer [2]. Some of the prospective clinical trials in immunotherapy with promising results were recently reported in advanced triple-negative breast cancer [3, 4].

$CD8^+$ cytotoxic T (Tc) cells have important roles in anti-tumoral immunity. Tc cells can kill tumor cells with granzymes and perforin and secrete interferon (IFN)-γ, which can increase the expression of MHC class I antigens by tumor cells [5]. Natural killer (NK) cells are innate lymphoid cells, have an intrinsic ability to detect and kill cancer cells [5]. Human NK cells can be divided into two main populations based on their relative expression of CD56 and CD16 molecules. $CD16^+CD56^{dim}$ NK cells constitute the majority of NK cells in peripheral blood and represent the main effector population while $CD16^-CD56^{bright}$ NK cells are predominantly found within lymphoid tissues [6]. While $CD16^+CD56^{dim}$ cells are more cytotoxic, $CD56^{bright}$ cells produce immunoregulatory cytokines such as interferon IFNγ and tumor necrosis factor (TNF)-α [6].

The programmed cell death ligand 1 (PD-L1) pathway, which is one of the immune checkpoints, plays a very important role in cancer immunotherapy [1, 7]. PD-L1 is a 40 kDa transmembrane protein expressed on epithelial cells, vascular endothelial cells, natural killer (NK) cells, macrophages, myeloid dendritic cells, and B cells [8]. The binding of PD-L1 to its receptor (PD-1) can regulate T-cell

activity and develop immune tolerance to its antigens [9, 10]. Similarly, the cancer cell can escape from the immune system by expressing PD-L1. It has been proven that PD-L1 is widely expressed in various types of cancer and increases tumor growth [10]. The relationship between some clinicopathological factors in breast cancer and PD-L1 expression has been reported in several studies [11, 12]. A meta-analysis revealed that high PD-L1 expression in breast tumors is a poor prognostic factor associated with lymph node metastasis, low nuclear grade, and negative estrogen receptor status [12].

T-cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT) expressed in NK and T cells, is a T-cell surface molecule as an immune checkpoint molecule that inhibits T-cell responses; however, its role in cancer is not well known. TIGIT, which belongs to the CD28 family, connects to CD226 and CD155, and binding of CD155 to TIGIT suppresses T-cell activation whereas binding to CD226 increases T-cell activation [13–15]. TIGIT expressions in peripheral blood mononuclear cells patients were significantly found to be higher in patients with breast cancer than healthy subjects [16].

Lymphocyte activation gene 3 (LAG-3) expressed on lymphocytes is a recently identified inhibitory receptor that is highly expressed in regulatory T (Treg) and anergic T cells [17]. MHC class II is the only known ligand for increased expression of LAG-3 in some epithelial cancers, and LAG-3/MHC class II interaction suppresses T cell responses. Inhibition of LAG-3/MHC class II interaction with mAbs increased tumor-specific $CD8^+$ T cell growth and cytokine production as demonstrated in some clinical immunotherapy trials in patients [18, 19]. In a study evaluating the relationship between breast cancer prognosis and LAG-3 expression, LAG-3 expression on tumor-infiltrating lymphocytes (TILs) was detected in 11% of tumors and was significantly associated with clinicopathological parameters such as young age, tumor size, high proliferation rate, HER-2 positivity [20].

T-cell immunoglobulin and Mucin domain-containing molecule 3 (TIM3) is specifically expressed on interferon-γ producing $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells, as well as Th17 cells, DCs (dendritic cells), monocytes, Tregs, mast cells, NK cells, and tumor infiltrated lymphocytes [21, 22]. It has been shown that high expression of TIM-3 on TILs was found to be significantly associated with clinicopathological features such as gender, age, lymph node metastasis, and TNM stage (p=0.015, 0.001, and 0.027, respectively) [23].

Despite increased complete pathologic response rates in locally advanced breast cancer following neoadjuvant chemotherapy (NAC), there are still a significant number of patients who have partial response or resistance to systemic chemotherapy. This prospective study aimed to determine immune checkpoint receptor expressions on TILs and to find any associations between tumor characteristics and immune checkpoints in patients with residual tumor after NAC.

Patients and methods

Between September 2018 and November 2019, 24 patients with locally advanced breast cancer (LABC) with residual tumor after NAC were included in this prospective study at the Breast Unit, Department of General Surgery, Istanbul Faculty of Medicine, Istanbul University. The approval of the ethics committee was obtained, and the informed consent form was signed by the patients who accepted to participate in the study. All of our patients belong to the Caucasian Turkish ethnic group. Patients older than 18 years with macroscopic residual tumor after neoadjuvant chemotherapy with the diagnosis of LABC were included in the study, whereas patients with a complete response to NAC, or pregnancy were excluded from the study. All patients were diagnosed with locally advanced breast cancer except 2 patients with oligometastatic bone metastases as proven in fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) before starting with NAC.

The majority of the patients (n=17, 70.8%) received 4 cycles adriamycin (60 mg/m^2) and cyclophosphamide (500 mg/m^2) plus 12 cycles of weekly paclitaxel (80 mg/m^2). Of the remaining patients, 1 (4.1%) had six cycles of 5-fluorouracil (500 mg/m^2), adriamycin (60 mg/m^2), and cyclophosphamide (500 mg/m^2), 1 (4.1%) had seven cycles adriamycin (60 mg/m^2) and cyclophosphamide (500 mg/m^2) plus four cycles of docetaxel (75 mg/m^2), 1 (4.1%) patient had eight cycles docetaxel followed by carboplatin, and 4 (16.7%) patients received anthracycline and taxanes based chemotherapy regimens with different protocols. All patients with HER2-neu positive disease additionally received trastuzumab therapy (2 mg/kg) with taxanes, and 1 of them had also pertuzumab as anti-HER2-neu targeted therapy in addition to the trastuzumab.

Isolation of TILs from tumor tissue. The surgical specimen was evaluated at the Department of Pathology for intraoperative examination, and tissue retrieval that was sent to the Immunology Laboratory. At least 1 cm^3 fresh tumoral tissue was obtained that was preserved in RPMI (Roswell Park Memorial Institute medium, Biological Industries, USA) on ice until processing in the immunology laboratory. TILs were isolated from tumor tissue obtained from the surgical material with a cell dissociation kit by using an MACS tumor separation device (Miltenyi Biotec, Germany). The tumor tissue was cleaned from necrotic areas and was cut into small pieces and transferred into the gentle MACS C Tube containing a mix of Enzymes H, R, and A (Tumor Dissociation Kit, human; Miltenyi Biotec, Germany). Tumor dissociation with the gentle MACS Dissociator was used according to the manufacturer's instructions. Mechanical dissociation was performed by a gentle MACS Dissociator, and the C Tube was rotated continuously for 30 min at 37 °C for three times to allow for enzymatic digestion. The obtained tissue suspension was passed through a cell strainer and washed with PBS (Sigma-Aldrich, USA). TILs were

further purified from Ficoll/Hypaque (Biochrom AG, Berlin, Germany) density gradient centrifugation.

Freshly isolated TILs from tumor material were evaluated for the presence of PD-1, CTLA-4, TIGIT, LAG-3, TIM-3 expression by flow cytometry as described below. Expressions of ICRs including PD-1, LAG-3, TIM-3, TIGIT, and CTLA-4 on $CD8^+$ T lymphocytes and NK cell subsets obtained from TILs were analyzed by using flow cytometry.

PD-1, TIGIT, LAG-3, CTLA-4, and TIM-3 expressions in TILs. Single-cell suspensions derived from breast tumor tissue were labeled fluorochrome-labeled mAbs (Biolegend, San Diego, CA, USA): anti-human CD223 (LAG-3)-FITC (clone 11C3C65, catalog number 369307), anti-human CD366 (TIM-3)-PerCP/Cy5.5 (clone F38-2E2, catalog number 345011), anti-human CD279 (PD-1)-APC/Cy7 (clone EH12.2H7, catalog number 329935), anti-human

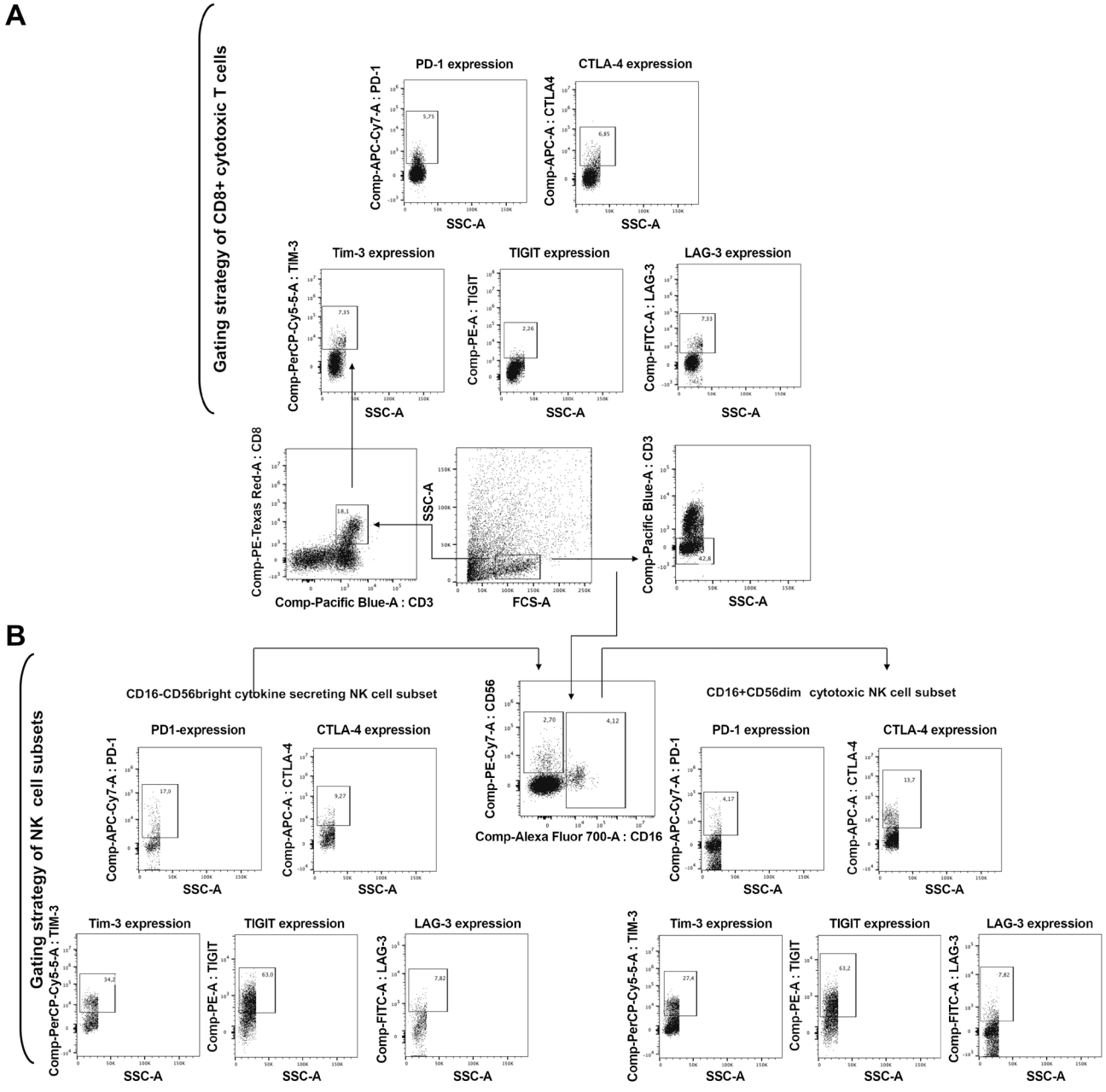

Figure 1. Gating strategy of $CD8^+$ T cells and NK cell subsets and immune checkpoint receptor expressions. Representative FACS dot plots from tumor tissue obtained from patients with locally advanced breast cancer were shown. Tumor-infiltrating lymphocytes were stained with anti-CD3, -CD16, -CD56 -CD8, -CTLA-4, -PD-1, -Tim-3, -LAG-3, and -TIGIT monoclonal antibodies and analyzed by flow cytometry. Gating was set on $CD3^+CD8^+$ cytotoxic T cells (A) and $CD3^-$ NK cells. The NK cell subsets were analyzed in the CD3 negative lymphocyte population and differentiated into, cytotoxic ($CD16^+CD56^{dim}$) and cytokine secreting ($CD16^-CD56^{bright}$) population based on the expression of CD56 and CD16 (B). Representative gating strategy of $CD8^+$ T cells, $CD16^+CD56^{dim}$, and $CD16^-CD56^{bright}$ NK cells and their PD-1, CTLA-4, LAG-3, TIGIT, and Tim-3 expression were also shown.

CD16-AlexaFlour700 (clone3G8, catalog number 302025), anti-human CD56-PECy7 (clone 5.1H11, catalog number 362507), anti-human TIGIT-PE (clone A15153G, catalog number 372723), anti-human CD3-Pacific blue (clone HIT3a, catalog number 300329), anti-human CD152 (CTLA-4)-APC (clone BNI3, catalog number 369625), anti-human CD8a PE/Dazzle (clone HIT8a, 300929) and incubated for 30 min at room temperature in the dark. The auto-fluorescent tube was used as an isotypic control for analysis. Following staining, cells were centrifuged with PBS solution once at 2000×g for 5 minutes. The cells were re-suspended with 500 µl of PBS with 1% paraformaldehyde and were analyzed with a FACSAriaII (BD Biosciences, San Jose, CA) running FACSDiva software. Data analysis was conducted with FlowJo™10.2 (Tree Star Inc., USA). The gating strategy of NK cell subsets and cytotoxic T cells and their ICR expressions are shown in Figure 1.

Pathological examination and immunohistochemical analysis. The tumors obtained from the surgical specimen were completely sampled for further evaluation at the Department of Pathology. The pathologic features of the tumors including tumor type, residual tumor size, presence of lymphovascular invasion (LVI), evaluation of tumor-infiltrating lymphocytes (host defense factor) and axillary lymph node status, and immunohistochemical findings regarding estrogen receptor, (ER, catalog number-ACA301A, B, C; clone SP1, 1:100 dilution; Biocare Concord, CA, USA) and progesterone receptor, (PR, catalog number-CRM325A, B, C; clone SP2, 1:400 dilution; Spring Pleasanton, California, USA), human epidermal growth factor receptor-2 (Her-2; catalog number-ACA342A, B; clone SP3, 1:200 dilution; Thermo Waltham, Massachusetts, USA) and Ki-67 (catalog number-ab166661, clone SP6, 1:100 dilution; Biocare Concord, California, USA) were assessed. The cut-off value for ER and PR positivity was at least 1% of tumor cells. Immunohistochemical analysis for HER-2 was scored according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines.

Table 1. Demographic, clinical, and pathological features of the patients.

Characteristics	%	Characteristics	%
Median Age (years, min-max)	49 (29-69)	N1	11 (45.8)
Age		N2	4 (16.6)
<50 years	13 (54.2)	N3	2 (8.3)
≥50 years	11 (45.8)	N1-N2-N3	17 (70.8)
Menopausal status		N0-N1	18 (75.0)
Pre-menopausal	13 (54.2)	N2-N3	6 (25.0)
Post-menopausal	11 (45.8)	Presence of EIC	
Regression level		Negative	12 (50.0)
Regression ≤ 20%	11 (45.8)	Positive	12 (50.0)
Regression > 20%	13 (54.2)	LVI	
cT stage		Negative	13 (54.2)
T1	0 (0)	Positive	11 (45.8)
T2	16 (66.6)	ER	
T3	1 (4.1)	Negative	10 (41.7)
T4	7 (29.1)	Positive	14 (58.3)
cN stage		PR	
N0	3 (12.5)	Negative	11 (45.8)
N1	11 (45.8)	Positive	13 (54.2)
N2	8 (33.3)	Ki-67	
N3	2 (8.3)	Median	50 (2-80)
ypT0-1		≥ 20%	9 (37.5)
< 2 cm	12 (50)	< 20%	15 (62.5)
≥ 2 cm	12 (50)	HER-2	
ypT stage		Negative	19 (79.2)
T0	2 (8.3)	Positive	5 (20.8)
T1	10 (41.6)	Molecular subtypes	
T2	8 (33.3)	Luminal A	8 (33.3)
T3	1 (4.1)	Luminal B	4 (16.6)
T4	3 (12.5)	Non-luminal HER2	3 (12.5)
ypN stage		Triple-negative	9 (37.5)
N0	7 (29.2)	Other	15 (62.5)

Abbreviations: EIC-Extensive Intraductal Component; LVI-Lymphovascular invasion; ER-Estrogen receptor; PR-Progesterone receptor; HER-2-Human epidermal growth factor receptor 2

Evaluation of chemotherapy response. Response to NAC was evaluated by "MD Anderson Cancer Center Residual Cancer Burden Index" (MDACC RCBI) [24, http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3]. The variables including a percentage of *in situ* tumor tissue, number of metastatic lymph nodes, and diameter of the largest lymph node metastasis were used to calculate the MDACC RCBI. These variables were entered into the MD Anderson Residual Cancer Burden Calculator to obtain a "residual cancer score from 0 (pathological complete response) to 3 (no response to NAC or chemotherapy-resistant).

In addition, the tumor regression rate measured microscopically in the tumor as regressional fibrosis was analyzed as <20% (poor response) and >20% (good response) to further evaluate the response to chemotherapy [25].

Statistical analysis. SPSS 17 (Statistical Package for Social Sciences; SPSS, Inc, Chicago, IL) program was used for statistical analysis. Descriptive statistical methods (mean, standard deviation, median, frequency, ratio, minimum, maximum) were used to evaluate the study data. Mann Whitney U test was used for comparing two groups of non-normally distributed variable variables, and Spearman correlation test was used for correlation of continuous variables with each other. A p-value ≤0.05 was considered significant.

Results

Demographic, clinical, and pathological characteristics of the patients. The study included 24 patients with LABC with residual tumor after NAC. The demographic and clinicopathological features of the patients are shown in Table 1. The median age of the patients was 49 years (29–69), 13 (54.2%) were under 50 years old and premenopausal, whereas 11 (45.8%) were postmenopausal. Of those, 13 (54.2%) have shown a significant tumor regression (>20%), whereas a partial response detected as tumor regression ≤20% was seen in the remaining cases (n=11, 45.8%). When pathological T and N stages were evaluated, 75% (n=18) of patients had pT1-T2, and 29.2% (n=7) of patients had pN0 stages. Presence of extensive intraductal component (EIC) and LVI were 50% (n=12) and 45.8% (n=11), respectively. Further, ER, PR, and HER-2 positivity rates were 58.3% (n=14), 54.2% (n=13), and 20.8% (n=5), respectively. The average Ki-67 value was 39.29±28.20%. The molecular subtypes of tumors revealed luminal type in half of the patients, whereas 37.5% (n=9) had triple-negative breast cancer.

Flow cytometric analyses of ICRs on TILs. Patients <50 years were more likely to express CTLA-4 on $CD8^+$ T lymphocytes compared to those ≥50 years (p=0.004, Figure 2, Table 2). In addition, patients with ypT3-4 tumors

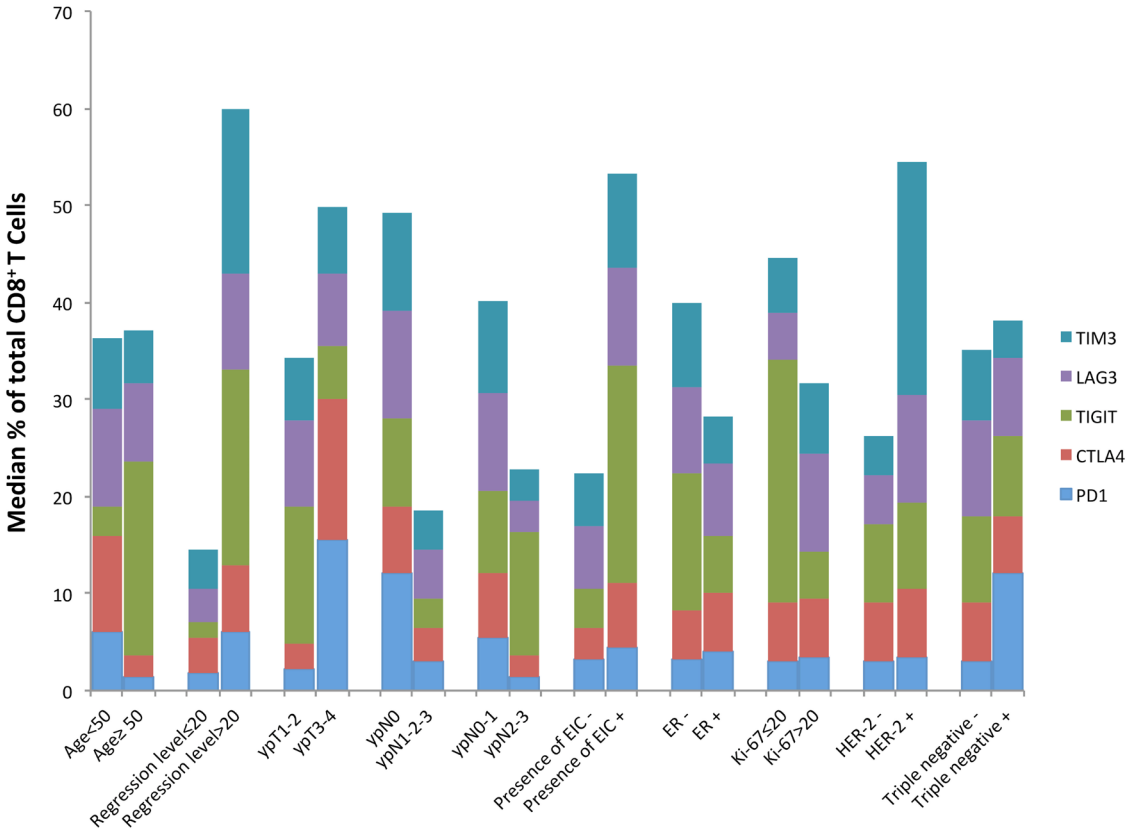

Figure 2. Flow cytometry data represented as the median percentage of $CD8^+$ T lymphocytes. TIM3, LAG3, TIGIT, CTLA-4, and PD1 expression of $CD8^+$ T cells were analyzed according to different parameters.

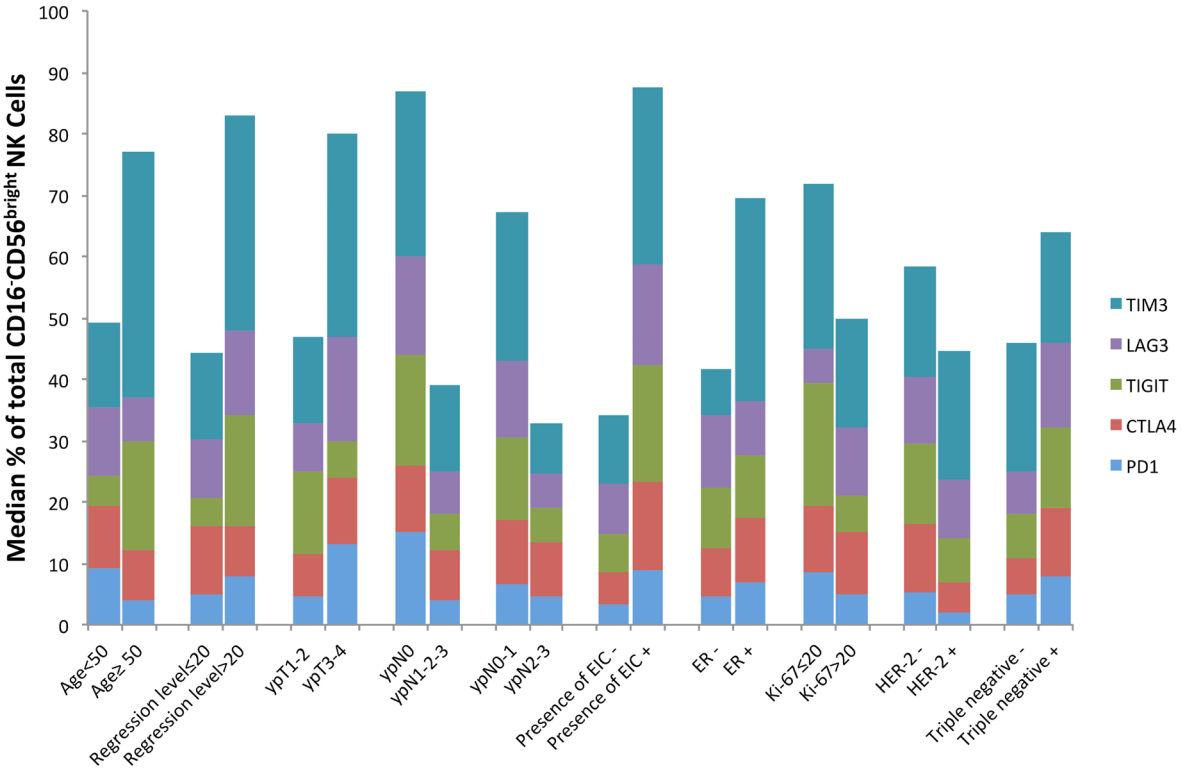

Figure 3. Flow cytometry data represented as the median percentage of $CD16^{-}CD56^{bright}$ cytokine secreting NK cells.

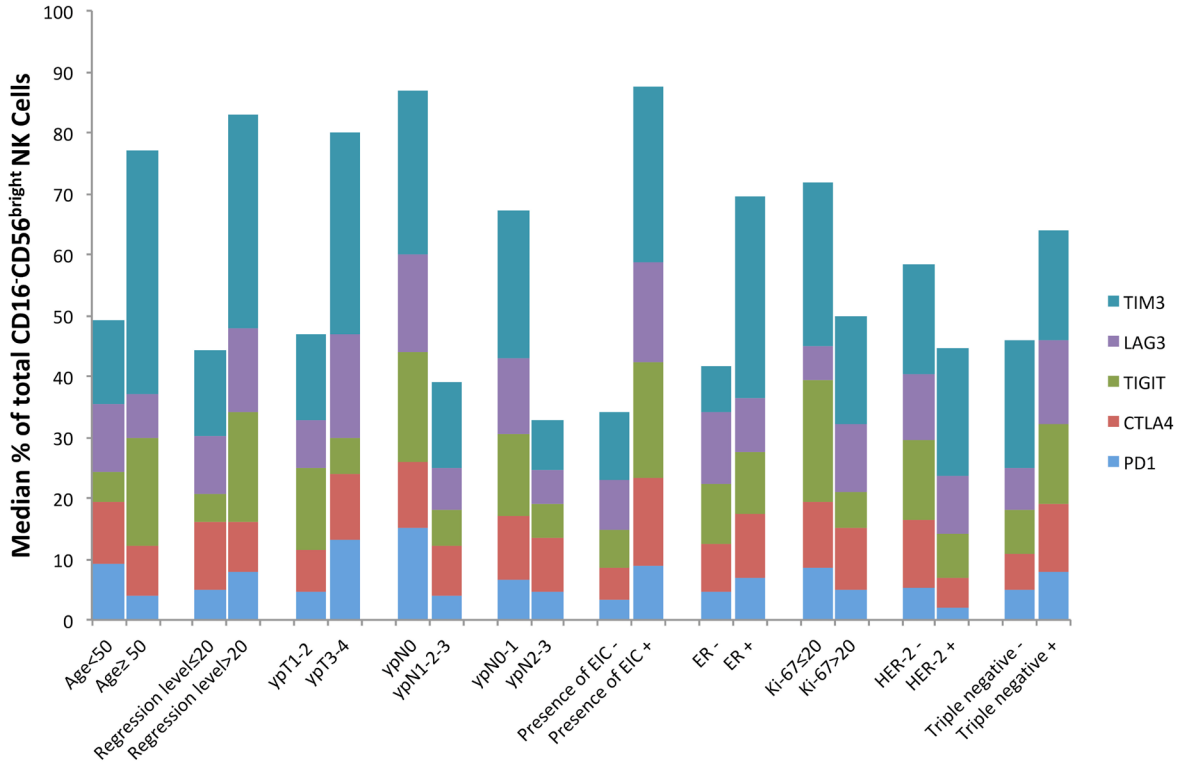

Figure 4. Flow cytometry data represented as the median percentage $CD16^{+}CD56^{dim}$ cytotoxic NK cells.

Table 2. Evaluation of CD8⁺ T lymphocytes by demographic, clinical and pathological characteristics.

Characteristics	CD8⁺ PD1		CD8⁺ CTLA4		CD8⁺ TIGIT		CD8⁺ LAG3		CD8⁺TIM3	
	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value
Age		0.20		0.004		0.66		0.64		0.62
<50 (n=13)	6 (0–46)		10 (2–27)		3 (0–54)		10 (1–50)		7.3 (1–54)	
≥50 (n=11)	1.34 (0–16)		2.24 (1–10)		20 (0–55)		8 (1–45)		5.5 (2–58)	
Regression level		0.24		0.88		0.01		0.18		0.06
≤20 (n=11)	1.9 (0–33)		3.54 (2–24)		1.7 (0–42)		3.36 (1–50)		4 (2–54)	
>20 (n=13)	6 (0–46)		7 (1–27)		20 (2–55)		10 (2–45)		17 (1–58)	
ypT		0.01		0.02		0.69		0.48		0.89
ypT1-2 (n=18)	2.17 (0–18)		2.7 (1–24)		14.1 (0–55)		8.9 (1–50)		6.4 (1–58)	
ypT3-4 (n=6)	15.5 (2–46)		14.5 (6–27)		5.5 (0–33)		7.5 (3–32)		6.88 (3–40)	
ypN		0.48		0.39		0.43		0.11		0.27
ypN0 (n=7)	12 (0–46)		7 (1–27)		9 (2–54)		11.2 (4–45)		10 (3–50)	
ypN1-2-3 (n=17)	3 (0–33)		3.54 (1–24)		3 (0–55)		5 (1–50)		4 (1–58)	
ypN		0.16		0.37		0.50		0.09		0.04
ypN0-1 (n=18)	5.5 (0–46)		6.5 (1–27)		8.6 (0–55)		10 (1–50)		9.5 (1–58)	
ypN2-3 (n=6)	1.45 (0–15)		2.12 (2–24)		12.7 (0–42)		3.33 (1–10)		3.13 (2–11)	
EIC		0.93		0.79		0.25		0.47		0.47
yes (n=12)	3.2 (0–46)		3.27 (1–27)		4 (0–28)		6.5(1–50)		5.35 (1–54)	
no (n=12)	4.5 (0–33)		6.5 (1–24)		22.5 (0–55)		10 (1–45)		9.75 (2–58)	
ER		0.35		0.81		0.86		0.62		0.91
negative (n=10)	3.2 (0–15)		5 (1–24)		14.1 (0–42)		9 (1–50)		8.65 (1–54)	
positive (n=14)	4 (0–46)		6 (1–27)		6 (0–55)		7.4 (1–32)		4.75 (2–58)	
Ki-67		0.98		0.86		0.12		0.31		0.57
≤20 (n=9)	3 (0–18)		6 (1–24)		25 (0–54)		5 (1–23)		5.5 (2–40)	
>20 (n=15)	3.4 (0–46)		6 (1–27)		5 (0–55)		10 (1–50)		7.3 (1–58)	
HER-2		0.89		0.97		0.69		0.07		0.04
negative (n=19)	3 (0–33)		6 (1–27)		8.2 (0–54)		5 (1–45)		4 (1–50)	
positive (n=5)	3.4 (1–46)		7 (1–12)		9 (1–55)		11 (5–50)		24 (5–58)	
Triple-negative		0.86		0.59		0.88		0.83		0.31
no (n=15)	3 (0–46)		6 (1–24)		9 (0–55)		9.8 (1–50)		7.3 (2–58)	
yes (n=9)	12 (0–33)		6 (1–27)		8.2 (2–42)		8 (1–45)		4 (1–50)	

Abbreviations: EIC-Extensive Intraductal Component, LVI-Lymphovascular invasion, ER-Estrogen receptor, HER-2-Human epidermal growth factor receptor 2

were more likely to have increased PD-1 (p=0.014) and CTLA-4 (p=0.018) expressions on CD8⁺ T cells in regards to those with ypT1-T2 (Figure 2, Table 2), and increased LAG-3 expression on $CD16^{-}CD56^{bright}$ NK cells (p=0.042, Figure 3, Table 3), respectively. Further, TIM-3 expression on CD8⁺ T cells was found to be increased in ypN0-1 (p=0.042) and HER2+ (p=0.043) compared to the ypN2-3 and HER2-negative cases, respectively (Figure 2, Table 2). Contrarily, PD-1 expression on $CD16^{-}CD56^{bright}$ NK cells were found to be decreased in patients with ypN+ than those with ypN– (p=0.022, Figure 3, Table 3).

In the analysis of cases according to ER and PR status, $CD16^{-}CD56^{bright}$ TIM-3 expression was found to be higher in ER-positive patients, but this association remained below the statistical significance (p=0.057, Figure 3, Table 3). In comparison with Ki-67 levels, $CD16^{+}CD56^{dim}$ TIGIT expression was found higher in those with Ki-67 ≤20%, but it did not reach the statistical significance (p=0.06, Figure 4, Table 4). Interestingly, TIGIT and LAG-3 expressions on $CD16^{+}CD56^{dim}$ NK cells, and PD-1 and CTLA-4 expressions on $CD16^{-}CD56^{bright}$ NK cells were found to be increased in patients with extensive intraductal component (p=0.024, p=0.003, p=0.032, p=0.013, respectively; Figures 3, 4; Tables 3, 4).

Notably, patients with a better response to NAC (tumor regression >20%) were more likely to express TIGIT in CD8⁺ T (p=0.014) and $CD16^{-}CD56^{bright}$ NK cells (p=0.003), respectively (Figures 2, 4; Tables 2, 3). Finally, pathological regression scores were significantly correlated with CTLA-4 expressions on $CD16^{+}CD56^{dim}$ NK cells by using Spearman correlation test (p=0.031).

Discussion

In this study, immune checkpoint receptor expressions on TILs were evaluated to determine their associations with

Table 3. Evaluation of CD16$^-$CD56^{bright} cytokine secreting NK cells by demographic, clinical, and pathological characteristics.

Characteristics	CD16$^-$CD56^{bright} PD1		CD16$^-$CD56^{bright} CTLA4		CD16$^-$CD56^{bright} TIGIT		CD16$^-$CD56^{bright} LAG3		CD16$^-$CD56^{bright} TİM3	
	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value	Median (Min–Max)	p-value
Age		0.27		0.64		0.12		0.82		0.32
<50 (n=13)	9.3 (0–21)		10 (2–22)		5 (0–63)		11 (0–70)		14 (0–70)	
≥50 (n=11)	4 (0–20)		8 (0–39)		18 (1–75)		7 (1–26)		40 (1–65)	
Regression level		0.62		0.84		0.003		0.79		0.17
≤20 (n=11)	5 (0–20)		11 (2–26)		4.8 (1–22)		9.5 (0–70)		14 (0–70)	
>20 (n=13)	8 (0–21)		8 (0–39)		18 (0–75)		14 (0–32)		35 (2–65)	
ypT stage		0.057		0.37		0.84		0.04		0.37
ypT1-2 (n=18)	4.5 (0–21)		7 (0–39)		13.4 (0–75)		8 (0–26)		14 (0–65)	
ypT3-4 (n=6)	13 (4–15)		11 (5–22)		6 (3–32)		17 (6–70)		33 (2–70)	
ypN		0.02		0.37		0.12		0.10		0.34
ypN0 (n=7)	15 (0–21)		11 (0–39)		18 (5–63)		16 (5–26)		27 (10–55)	
ypN1-2-3 (n=17)	4 (0–14)		8 (2–38)		6 (0–75)		7 (0–70)		14 (0–70)	
ypN		0.59		0.62		0.20		0.22		0.17
ypN0-1 (n=18)	6.7 (0–21)		10.5 (0–39)		13.4 (0–75)		12.5 (0–70)		24 (0–70)	
ypN2-3 (n=6)	4.5 (2–14)		9 (2–21)		5.45 (1–20)		5.7 (1–18)		8 (1–50)	
EIC		0.03		0.01		0.11		0.07		0.19
no (n=12)	3.4 (0–20)		5 (0–26)		6.5 (0–30)		8 (0–26)		11.35 (0–59)	
yes (n=12)	8.85 (2–21)		14.5 (2–39)		19 (3–75)		16.35 (2–70)		29 (2–70)	
ER		0.30		0.95		0.41		0.81		0.06
negative (n=10)	4.5 (0–20)		8 (0–39)		9.9 (1–29)		11.75 (0–26)		7.35 (0–55)	
positive (n=14)	6.9 (2–21)		10.5 (2–38)		10 (0–75)		9 (0–70)		33 (2–70)	
Ki-67		0.72		0.59		0.28		0.44		0.81
≤20 (n=9)	8.4 (2–21)		11 (2–38)		20 (0–63)		5.39 (0–32)		27 (1–54)	
>20 (n=15)	5 (0–20)		10 (0–39)		6 (3–75)		11 (0–70)		18 (0–70)	
HER-2		0.39		0.43		0.83		0.94		0.91
negative (n=19)	5.4 (0–21)		11 (0–39)		13 (0–63)		11 (0–70)		18 (1–70)	
positive (n=5)	2 (0–20)		5 (2–26)		7 (3–75)		9.5 (0–26)		21 (0–65)	
Triple-negative		0.49		0.35		0.83		0.12		0.40
no (n=15)	5 (0–21)		6 (2–38)		7 (0–75)		7 (0–32)		21 (0–65)	
yes (n=9)	8 (0–15)		11 (0–39)		13 (3–29)		14 (6–70)		18 (2–70)	

Abbreviations: EIC-Extensive Intraductal Component; LVI-Lymphovascular invasion; ER-Estrogen receptor; HER-2-Human epidermal growth factor receptor 2

the demographic, clinical, and pathological characteristics of patients following NAC. In this prospective clinical study, immune checkpoint expressions were highly expressed on TILs in locally advanced breast cancer with residual tumor following NAC. Differential immune checkpoint receptor expressions were detected on TILs of patients with advanced-stage breast cancer and younger <50 years, as well as in patients with a better response to chemotherapy and extensive intraductal component.

The relationship between clinicopathological factors and PD-L1 expression in breast cancer has been investigated in many studies [12, 26, 27]. In a meta-analysis, PD-L1 expression in breast tumors was found as a poor prognostic factor associated with lymph node metastasis, low nuclear grade, and negative estrogen receptor status [12]. Similarly, in our study, PD-1 expression on cytotoxic T lymphocytes in patients with ypT3-T4 after NAC was found to be higher than ypT1-2. Contrarily, PD-1 levels expressed on CD16$^-$CD56^{bright} NK cells in metastatic lymph nodes were lower than non-metastatic lymph nodes. In a study, PD-L1 expression was found to be increased in high-grade tumors in 180 patients who received NAC for stage II–III invasive breast carcinoma [26]. PD-1 and PD-L1 expressions were also found to be associated with high TIL-rate ($p<0.0001$), tumor molecular subtype, and triple-negative tumors ($p<0.0001$).

In another study by Buisseret et al., TIL density, PD-1, PD-L1 levels were evaluated by flow cytometry and immunohistochemical evaluation [27]. High PD-1/PD-L1 expression, high TIL density, and increased CD4$^+$ T and B cell infiltration were found to be associated with more aggressive tumor characteristics (Ki-67 height, hormone receptor

Table 4. Evaluation of CD16+CD56dim cytotoxic NK cells by demographic, clinical and pathological characteristics.

Characteristics	$CD16^+CD56^{dim}$ PD1		$CD16^+CD56^{dim}$ CTLA4		$CD16^+CD56^{dim}$ TIGIT		$CD16^+CD56^{dim}$ LAG3		$CD16^+CD56^{dim}$ TIM3	
	Median (Min-Max)	p-value	Median (Min-Max)	p-value	Median (Min-Max)	p-value	Median (Min-Max)	p-value	Median (Min-Max)	p-value
Age		0.71		0.12		0.95		0.75		0.95
50 (n=13)	4.3 (1–52)		16 (0–45)		4 (2–29)		6 (0–35)		10 (2–34)	
≥50 (n=11)	7 (0–31)		11 (0–20)		10 (0–35)		7 (0–30)		15 (0–30)	
Regression level		0.73		0.23		0.21		0.84		0.09
≤20 (n=11)	4 (0–52)		19 (0–45)		4 (0–20)		5.4 (0–35)		7 (0–34)	
>20 (n=13)	6 (0–31)		11 (0–31)		10 (0–35)		7 (0–30)		15 (2–30)	
ypT stage		0.32		0.30		0.81		0.19		0.71
ypT1-2 (n=18)	4.15 (0–31)		11.5 (0–31)		6.5 (0–35)		5.2 (0–30)		15 (0–30)	
ypT3-4 (n=6)	6 (2–52)		15.5 (4–45)		4.5 (2–29)		17 (0–35)		10 (3–34)	
ypN stage		0.77		0.63		0.82		0.77		0.11
ypN0 (n=7)	6 (0–31)		13 (0–19)		10 (0–23)		14 (0–30)		22 (10–30)	
ypN1-2-3 (n=17)	4.3 (0–52)		12 (0–45)		6 (0–35)		5.4 (0–35)		10 (0–34)	
ypN stage		0.59		0.39		0.55		0.69		0.14
ypN0-1 (n=18)	5.15 (0–52)		12 (0–31)		5.65 (0–35)		8 (0–35)		15.5 (2–30)	
ypN2-3 (n=6)	5 (0–25)		18 (2–45)		6 (1–20)		6.2 (0–33)		5 (0–34)	
EIC		0.20		0.09		0.02		0.003		0.09
no (n=12)	4 (0–30)		7 (0–31)		2 (0–35)		1.73 (0–29)		10 (0–26)	
yes (n=12)	9.5 (0–52)		15.3 (2–45)		10.8 (1–29)		14.5 (0–35)		17.5 (2–34)	
ER		0.66		0.35		0.56		0.56		0.81
negative (n=10)	4.15 (0–31)		9.5 (0–30)		6.5 (0–23)		5.2 (0–30)		11.5 (0–30)	
positive (n=14)	6.50 (52–)		14.8 (2–45)		5 (0–35)		8.5 (0–35)		13 (2–34)	
Ki-67		0.98		0.47		0.06		0.53		0.51
≤20 (n=9)	4 (1–30)		12 (2–45)		11.6 (1–35)		7 (0–33)		15 (0–34)	
>20 (n=15)	6 (0–52)		13 (0–30)		3 (0–23)		6 (0–35)		10 (2–30)	
HER-2		0.91		0.25		0.86		0.62		0.97
negative (n=19)	6 (0–52)		13 (0–45)		6 (0–35)		7 (0–35)		11 (0–34)	
positive (n=5)	4 (1–16)		6 (0–19)		7.3 (2–18)		5 (0–29)		15 (2–26)	
Triple-negative		0.98		0.40		0.19		0.86		0.63
no (n=15)	4 (0–30)		11 (0–45)		7.3 (1–35)		7 (0–33)		15 (0–34)	
yes (n=9)	6 (0–52)		15 (0–30)		3 (0–23)		6 (0–35)		10 (2–30)	

Abbreviations: EIC-Extensive Intraductal Component; LVI- Lymphovascular invasion; ER-Estrogen receptor; HER-2-Human epidermal growth factor receptor 2

negativity) in this study. Similar studies suggested that the expressions of ICRs were found to be associated with poor prognosis [28]. In a meta-analysis conducted by Wang et al., PD-L1 overexpression was found in 25.8% of patients with breast cancer [28]. In addition, PD-L1 positivity were found related with invasive ductal cancer (p=0.037), high tumor grade (p=0.0001), ER-negativity (p=0.0001), PR-negativity (p=0.0001), HER-2 positivity (p=0.001), and poor prognostic factors such as aggressive molecular subtypes including HER-2 positive and basal-like (p=0.0001). In order to clarify this issue, clinical and experimental studies with long-term follow-up survival information of different patient groups are needed.

According to the study by Solinas et al. using gene expression, immunohistochemistry, and flow cytometry in patients with breast cancer, LAG-3 expression was found to be increased in basal type breast cancers than other subtypes, in patients with HER-2 positive breast cancer than in the luminal subgroups, and finally in luminal B than in luminal A patients [29]. In the same study, LAG-3 was found to be low on $CD4^+$ and $CD8^+$ TILs, and LAG-3 positivity rates on $CD4^+$ or $CD8^+$ TIL correlated positively with the percentage of Ki-67, stromal TIL, global TIL, $CD4^+$, $CD8^+$, and $CD20^+$. In the flow-cytometric analysis in our study, LAG-3 expression in $CD16^+CD56^{dim}$ NK cells was found to be higher in patients with EIC-positive tumors compared to those with EIC-negative tumors.

In the study reported by Zhang et al., the expression of TIM-3 expression was found to be higher in patients with age over 45 years (p=0.015), lymph node involvement (p=0.001), and high TNM stage (p=0.027) [23]. In this study, the rate of $CD8^+$ T cells infiltrating the tumor was found higher in

patients with lymph node metastasis, high TNM stage, and high Ki-67 level [23]. Contrarily, TIM-3 expression on cytotoxic T lymphocytes of patients with ypN2-N3 was lower than patients with ypN0-N1 in our study. In addition, we found higher TIM-3 expression on cytotoxic T lymphocytes in HER-2 positive patients compared to those in HER-2 negative cases.

Elashi et al. examined the PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, and PD-L1 levels in peripheral blood mononuclear cells of breast cancer patients (n=31) detecting higher TIGIT and PD-L1 and lower LAG3 expressions compared to healthy controls [16]. Furthermore, in another study by Nair et al., PD-1, CTLA-4, TIM-3, and LAG-3 levels were found to be higher in breast tumor tissues compared to normal breast tissue among patients with breast cancer [30].

There are few studies about the expression of TIGIT in cancer. Duan et al. examined the healthy tissue samples adjacent to the tumor tissue of 77 hepatocellular cancer (HCC) patients, and the expression of TIGIT and CD155 in cancerous tissue was found to be significantly higher [31]. Similarly, few reports are available on whether ICRs could predict response to NAC. In our study, TIGIT expression on both $CD8^+$ T cells (p=0.014) and $CD16^-CD56^{bright}$ NK cells (p=0.03) was significantly found to be increased in patients with tumor regression rates higher than 20% after NAC. Therefore, response to NAC may be better in patients with tumors expressing high TIGIT levels. Furthermore, NAC itself causes changes in the tumor microenvironment affecting the immune response causing increased expression of ICRs. These results may suggest that targeted therapies developed against these receptors can be combined with chemotherapy to increase pathologic complete response rates to NAC. Larger clinical studies are needed to clarify this issue.

Interestingly, PD-1, CTLA-4, LAG-3, and TIGIT expressions on NK cells were detected to be increased in patients with EIC compared to those without EIC. In concordance with this finding, Del Alcazar and colleagues evaluated the effect of the immune escape mechanism in the process of transformation from *in situ* ductal carcinoma to invasive ductal carcinoma in breast cancer. In this study, they showed that TIGIT expression was higher in triple-negative *in situ* cases compared to the invasive ductal cancer group, similar to our findings [32]. Unlike TIGIT, PD-L1 expression was significantly higher in invasive ductal carcinoma than *in situ* cancer. These results suggest that immune escape, which is an important step in invasive tumor development, may be related to different ICRs [32].

In the study of Xu et al., it has been shown that CD112R and TIGIT blockade increased NK cell activity of breast cancer patients *in vitro* when combined with trastuzumab [33]. These results suggest that such targeted treatment combinations will become more frequent in the future. In our study, immune checkpoint receptor expressions on NK cells were investigated. In a prospective study by Kim et al., immune checkpoint gene expressions including PDCD1 (PD-1), CD274 (PD-L1), CD276 (B7-H3), CTLA-4, IDO1, LAG-3, VTCN1, HAVCR2, and TNFRSF4 (OX40) and HAVCR2 were reported to be high in a HER2-positive metastatic patient with pathologic complete response following taxanes combined with anti-HER-2 treatment [34]. However, hormone receptor status and breast cancer molecular subtypes did not affect the expression of ICRs suggesting taxane therapy can modify the cancer-immune environment [34]. It was also found that HER-2 expression level positively correlated with the level of ICRs in patients with metastatic breast cancer [34]. Our study also showed increased TIM-3 expression on $CD8^+$ T cells in patients with HER-2 positivity. These results also suggest that mAbs directed to new generation ICRs could be used in combination with anti-HER-2 therapy and/or with anti-PD-L1 and anti-CTLA-4 therapies that may increase the NAC response.

Triple-negative breast cancer accounted for around one-third of the patients in this study, which was much larger than the average since TNBC accounts for about 10–15% of all breast cancers [35]. In the present study, patients who were consecutively referred to neoadjuvant treatment between September 2018 and November 2019 and who had a partial response or were completely unresponsive with residual breast tumor were included in the study. Therefore, this situation might be entirely coincidental since the total number of cases is low.

The strength of this study is that the flow cytometric analysis of various ICRs is the first study in the literature to examine the new generation ICRs such as TIGIT, LAG-3, and TIM along with PD-1 and CTLA-4. The weakness of this study is the small number of patients in each group.

In conclusion, immune checkpoint receptors including the new generation TIM-3, LAG-3, and TIGIT are highly expressed in residual tumors following NAC especially in patients with poor prognostic factors. Therefore, new evolving therapies using inhibitory mAbs directed to TIM-3, LAG-3, and TIGIT could be also be considered in locally advanced breast cancers expressing these ICRs.

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