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ORIGINAL ARTICLE

Expression of the immune checkpoint VISTA in breast cancer

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Abstract

V-domain Ig suppressor of T cell activation (VISTA) is a novel immune checkpoint that is an emerging target for cancer immunotherapy. This study aimed to investigate the expression of VISTA and its association with clinicopathologic parameters as well as with the key immune markers including programmed cell death-1 (PD-1) and PD-1 ligand-1 (PD-L1) in invasive ductal carcinoma (IDC) of the breast. Immunohistochemistry was used to detect VISTA, PD-1, PD-L1, and CD8 in tissue microarrays from 919 patients with IDC ($N=341$ in the exploratory cohort and $=578$ in the validation cohort). VISTA was expressed on the immune cells of 29.1% (267/919) of the samples and on the tumor cells of 8.2% (75/919). VISTA was more frequently expressed in samples that were estrogen receptor-negative, progesterone receptor-negative, human epidermal growth factor receptor 2-positive, poorly differentiated, human epidermal growth factor receptor 2-enriched, and consisting of basal-like tumors. VISTA on immune cells correlated with PD-1, PD-L1, stromal CD8, and tumor-infiltrating lymphocyte expression and was an independent prognostic factor for improved relapse-free and disease-specific survival in patients with estrogen receptor-negative, progesterone receptor-negative, and basal-like IDC. These findings support therapeutic strategies that modulate VISTA expression, perhaps in combination with PD-1/PD-L1 blockade, in human breast cancer immunotherapy.

Keywords VISTA · Breast cancer · Cancer immunology · Immune checkpoint · Prognosis

Abbreviations

AJCC	American Joint Committee on Cancer
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CK	Cytokeratin
DSS	Disease-specific survival
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
IC	Immune cell
IDC	Invasive ductal carcinoma
LAG-3	Lymphocyte activation gene 3
PR	Progesterone receptor
REMARK	Reporting Recommendations for Tumor Marker Prognostic Studies
RFS	Relapse-free survival
TCGA	The Cancer Genome Atlas
TC	Tumor cell
TIM-3	T-cell Immunoglobulin and mucin domain-containing molecule 3
TILs	Tumor infiltrating lymphocytes
TMA	Tumor tissue microarray
VISTA	V-domain Ig suppressor of T-cell activation

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Introduction

Immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA4), programmed cell death-1 (PD-1), and/or its ligand PD-L1 have been shown to benefit patients with a variety of solid tumors, especially

those with immunogenic cancers such as melanoma and lung cancer [1]. However, even among patients with potentially immunogenic cancers, only a relatively small proportion benefit from immune checkpoint inhibitors. One hypothesis that explains the patients' resistance to these therapies is the activation of alternative immune checkpoints, which dampen T-cell responses and contribute to severe T-cell exhaustion. The modulation of additional immune checkpoints, such as V-domain Ig suppressor of T cell activation (VISTA), may achieve better clinical outcomes; therefore, such molecules are currently being actively researched [2].

VISTA, also known as PD-1H, VSIR, and c10orf54, is an immune checkpoint receptor discovered in 2011 that is expressed on tumor-infiltrating lymphocytes (TILs) and a variety of immune cells (ICs) including macrophages and T cells. VISTA promotes the suppression of T cell activation, proliferation, and cytokine production [3]. VISTA is distinct from PD-1 and CTLA4 in that it may act as both a ligand (by binding the co-inhibitory receptor P-selectin glycoprotein ligand-1 in acidic conditions) and a receptor (by binding the ligand V-set and immunoglobulin domain containing protein 3) when regulating immune responses [4, 5]. Moreover, its expression was shown to be elevated after anti-CTLA4 therapy in patients with prostate cancer, suggesting its potential involvement in a resistance mechanism [6]. Furthermore, preclinical data indicated that VISTA blockade releases T cell effector functions and synergizes with other immune checkpoint inhibitors such as anti-PD-L1 in colon cancer models [7]. PD-L1 and VISTA inhibition suppressed tumor growth in preclinical models and also promoted T cell activation in a phase I study [8]. Taken together, VISTA may represent a novel, promising target for cancer immunotherapy. Previous studies have investigated the expression of VISTA in human cancers such as lung cancer, gastric cancer, and gestational trophoblastic neoplasia [9–11]; they found that the relationship between VISTA and patient outcomes varies according to the type of cancer. VISTA expression on tumor-infiltrating inflammatory cells correlates with poor disease-specific survival in patients with primary cutaneous melanoma [12], but is associated with improved overall survival (OS) in patients with early-stage esophageal adenocarcinoma [13].

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related deaths in women worldwide [14]. Invasive ductal carcinoma (IDC) is the most common type of breast cancer, as it accounts for approximately 80% of all such cancers. It is generally considered one of the least immunogenic tumors; triple-negative breast cancer and human epidermal growth factor receptor 2 (HER2)-positive breast cancers are relatively more immunogenic than other types of breast cancer [15]. Checkpoint inhibitors have previously been shown to be effective in patients with triple-negative breast cancer whose tumors are PD-L1-positive [16].

However, little is known about the expression and clinical significance of VISTA in breast cancer, and the presence of potential links between PD-1, PD-L1, and VISTA expression remains unexplored. The aim of the present study was to assess VISTA expression as well as its association with clinicopathologic parameters and biomarkers such as PD-1/PD-L1 and CD8 in IDC.

Materials and methods

Study cohorts and tissue microarray (TMA) construction

The exploratory cohort comprised commercialized TMAs purchased from Shanghai Outdo Biotech Co. Ltd. (panel HBreD139Su01, HBreD140Su03, and HBreD150Su02). The TMAs consisted of 429 cores (1.5 mm diameter) from 429 patients with stage I–III breast cancer who underwent surgeries between 2001 and 2011. The validation cohort comprised 686 female patients with stage I–III breast cancer who were treated at Peking Union Medical College Hospital (Beijing, China) between 2014 and 2015. In the validation cohort, representative areas with mixed epithelial tumor tissue and tumor-related stroma were marked on the hematoxylin and eosin (HE)-stained slide and sampled for the TMA blocks. TMAs with one 2 mm core per case were constructed using a tissue microarray instrument. Patients with primary IDC were sought for this study, and the following patients were excluded from both the exploratory and validation cohorts: Those with invasive lobular carcinoma, mixed invasive ductal and lobular carcinoma, or special histological types such as invasive micropapillary carcinoma and mucinous carcinoma; those who received neoadjuvant chemotherapy before surgery; and those with inadequate formalin-fixed and paraffin-embedded tissue blocks or TMA cores with tumor and stromal contents < 5%.

The study was conducted according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [17] and conformed to the ethical standards set forth in the Declaration of Helsinki and in national and international guidelines. The study was approved by the Institutional Review Board of Peking Union Medical College Hospital. Given the retrospective study design and analysis of clinical data, written consent was formally waived by the Ethics Committee of Peking Union Medical College Hospital.

Evaluation of TILs

TILs present in full HE-stained sections that were derived from blocks used for TMA construction in the validation cohort were evaluated based on the recommendations of the International TILs Working Group 2014 [18]. TILs were assessed as a continuous parameter in 5% increments. For analysis, stromal TILs were classified into < 10%, 10–50%, and > 50% categories as described in a previous study [19].

Immunohistochemistry

Immunohistochemistry (IHC) was performed using our laboratory protocol as described previously [11, 20]. Briefly, 4-μm TMA serial sections were deparaffinized and subjected to heat-induced epitope retrieval with 10 mM sodium citrate (pH 6.0) at 95℃ for 20 min. The endogenous peroxidase activity was quenched using a 0.3% hydrogen peroxide solution. TMA sections were incubated with primary antibodies against VISTA, PD-L1, PD-1, and CD8, the details and dilutions of which are shown in Supplementary Table 1. Human tonsil and placenta tissues treated with primary antibodies were used as positive controls, while the same tissues without primary antibodies comprised the negative controls. All the slides were stained using an automatic IHC staining instrument (BOND-III; Leica Biosystems) according to the manufacturer's instructions.

Immunostaining procedures for estrogen receptor (ER), progesterone receptor (PR), HER2, Ki67, epidermal growth factor receptor (EGFR), and cytokeratin (CK) 5/6 have been well-validated during routine clinical practice at Peking Union Medical College Hospital, as has fluorescence in situ hybridization for HER2 expression. These methods were described previously [21, 22].

Evaluation of immunostaining

The immunostaining was assessed independently by two pathologists who were blinded to the patients' clinical outcomes. In case of disagreement, both pathologists reexamined the slides and reached a consensus. PD-L1 scoring is currently controversial; in this study, we used the method of Schmid et al. [16] whereby scoring is based on the percentage of PD-L1-expressing ICs with respect to the total tumor area. Consistent with Schmid et al.'s results [16], we found that PD-L1 expression was more prevalent on ICs than on breast tumor cells (TCs), while samples that exhibited PD-L1 expression on TCs usually expressed this ligand on ICs as well. Therefore, we evaluated PD-1 and PD-L1 expressions on stromal tumor-infiltrating ICs; PD-L1 and PD-1 were defined as positive if ≥ 1% of the stromal ICs were positive, as described in Schmid et al.'s study [16]. The percentages of $CD8^+$ lymphocytes among the nucleated cells in the stromal compartments (stromal CD8) were assessed and recorded as a continuous parameter. VISTA expression was evaluated on TCs and stromal tumor-infiltrating ICs separately. TCs were considered VISTA-positive if at least 5% of these cells per core had membranous and/or cytoplasmic staining. The percentages of VISTA-expressing IC were compared to those of tumor-associated ICs in the stromal compartments; stromal ICs with ≥ 5 VISTA staining were defined as VISTA-positive, as described in previous study [13].

The statuses of ER, PR, and HER2 in the exploratory cohort were determined by Shanghai Outdo Biotech Co. Ltd. For the validation cohort, ER and PR were classified as positive if at least 1% of TCs expressed these proteins unequivocally at any intensity. HER2 was defined as positive if more than 10% of TCs exhibited membrane staining scores of 3 +; samples with equivocal HER2 expression were tested using fluorescence in situ hybridization for confirmation [23, 24]. Intrinsic breast cancer subtypes in the validation cohort were determined using immunohistochemistry for ER, PR, HER2, Ki67, EGFR, and CK5/6. Luminal A was defined as ER + (≥ 1%) or PR + (≥ 1%), HER2–, and low Ki67(< 14%); luminal B was defined as ER + (or PR +) and HER2– or high Ki67 (≥ 14%); HER2-enriched (HER2E) was defined as HER +, ER–, and PR–; and basal-like was defined as ER–, PR–, and HER2– with either EGFR + or CK5/6 +.

Kaplan–Meier plotter analysis

To analyze the prognostic value of mRNA from the VISTA-encoding gene *C10orf54* in breast cancer, we performed survival analysis using the Kaplan–Meier plotter (www.kmplot.com), which contains gene expression data and patients' survival information which derived from the Gene Expression Omnibus, European Genome-phenome Archive, and TCGA. Patient samples were split into two groups (high vs. low expression) according to the most optimal cutoff of *C10orf54* mRNA levels, which was automatically determined by the Kaplan–Meier plotter [25].

Statistical analysis

All statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows (version 20.0; IBM Corp.). The chi-squared test was used to evaluate the relationship between VISTA expression and categorical variables. The Spearman rank correlation test was used to determine the correlation between continuous variables. Relapse-free survival (RFS) was defined as the time from the date of surgery to date of first local, regional, and/or distant relapse. Disease-specific survival (DSS) was defined as the time from the date of surgery to that of death caused by breast cancer. Survival curves were plotted using the

Kaplan–Meier method and compared using the log-rank test. To identify indicators of survival, we used a Cox proportional hazards model in which variables with a *p*-value of < 0.25 on univariate analyses were subjected to multivariate analysis; T and N stages were not included in these multivariate analyses as the American Joint Committee on Cancer (AJCC) stage was based on a combination of T and N stages.

Results

Expression of VISTA in IDC

The exploratory cohort comprised 341 patients with a median age of 57 years (range, 29–86 years); sixty-four patients were diagnosed with AJCC stage I, 181 with stage II, and 96 with stage III. The patients' clinicopathological characteristics are summarized in Supplementary Table 2. The validation cohort included 578 patients with a median age of 50 years (range, 22–83 years); these patients' detailed clinicopathological features, intrinsic molecular subtypes, stromal TILs, and adjuvant therapies are summarized in Supplementary Table 3.

VISTA was expressed on both ICs and TCs and exhibited a cytoplasmic/membranous staining pattern (Fig. 1a, b). In the exploratory cohort, 106 samples (31.1%) had VISTA-positive ICs while 20 (5.9%) had VISTA-positive TCs. In the validation cohort, 161 (27.9%) showed VISTA-positive ICs, while 55 (9.7%) had VISTA-positive TCs. There were no significant differences between the two cohorts with respect to VISTA expression on ICs and TCs. Altogether, positive VISTA expression on ICs was observed in 29.1% of the samples (267/919) and its expression on TCs was observed in 8.2% (75/919).

Associations between VISTA, immune markers, and clinicopathological parameters in IDC

In both the exploratory and validation cohorts, positive expression of VISTA on ICs was observed significantly more frequently in ER-negative, PR-negative, and HER2-positive samples (Supplementary Tables 2 and 1). Moreover, VISTA expression on ICs was significantly associated with younger age (< 50 years), poor differentiation, triple-negative status, and a high Ki67 proliferation index (Table 1). VISTA expression was associated with the intrinsic molecular subtype: VISTA was positive in 42.6% of samples with HER2E and 43.1% of those with basal-like subtypes, but its expression was significantly lower in samples with luminal A and luminal B types (10.1% and 23.4%, respectively; Table 1).

Positive expression of VISTA on ICs was significantly associated with PD-1-positivity, PD-L1-positivity,

Table 1 Expression of VISTA on the immune cells of patients with invasive ductal carcinoma of the breast (validation cohort, $N = 578$) and their clinical characteristics

Parameters	VISTA on ICs		
	Negative N	Positive N (%)	*p*-value
Age			0.022
< 50 years	210	64 (23.4)	
≥ 50 years	207	97 (31.9)	
Tumor stage			0.973
pT1	193	71 (26.9)	
pT2	201	81 (28.7)	
pT3	18	7 (28.0)	
pT4	5	2 (28.6)	
Differentiation			< 0.001
Poor	124	83 (40.1)	
Moderate	254	72 (22.1)	
Well	39	6 (13.3)	
Lymph node			0.838
pN0	188	76 (28.8)	
pN1	118	43 (26.7)	
pN2	45	20 (30.8)	
pN3	66	22 (25.0)	
AJCC stage			0.838
I	114	42 (26.9)	
II	188	77 (29.1)	
III	115	42 (26.8)	
ER			< 0.001
Negative	111	90 (44.8)	
Positive	306	71 (18.8)	
PR			< 0.001
Negative	142	97 (40.6)	
Positive	275	64 (18.9)	
HER2			0.001
Negative	327	105 (24.3)	
Positive	90	56 (38.4)	
Triple-negative			< 0.001
No	349	103 (22.8)	
Yes	68	58 (46.0)	
Ki67			< 0.001
< 14%	123	16 (11.5)	
≥ 14%	294	145 (33.0)	
Subtypes			< 0.001
Luminal A	107	12 (10.1)	
Luminal B	203	62 (23.4)	
HER2E	39	29 (42.6)	
Basal-like	62	47 (43.1)	
Unknown	6	11 (-)	

VISTA V-domain Ig suppressor of T-cell activation; *IC* immune cell; *AJCC* American Joint Committee on Cancer; *ER* estrogen receptor; *PR* progesterone receptor; *HER2(E)* human epidermal growth factor receptor 2(-enriched)

CD8-high status (≥ 10%), and stromal TILs (Supplementary Table 2 and Table 2). Sixty-four of the PD-L1-negative samples (14.6%) were VISTA-positive, whereas 43 of the VISTA-negative samples (10.3%) showed PD-L1 staining; as such, 107 samples (18.5%) showed complementary expression of VISTA and PD-L1. Moreover, the percentage of VISTA-positive ICs showed a significant positive correlation with that of stromal CD8-positive TILs (Spearman's rank correlation = 0.557, $p < 0.001$) and total stromal TILs (Spearman's rank correlation = 0.480, $p < 0.001$) in the validation cohort. However, the positive expression of VISTA on TCs was observed significantly more frequently among samples in which the ICs were VISTA-negative ($p = 0.046$). Nine samples were VISTA-positive on both ICs and TCs, 371 were VISTA-negative on both ICs and TCs, 152 were VISTA-positive on ICs but negative on TCs, and 46 were VISTA-positive on TCs but negative on ICs. Representative images of VISTA, TILs, PD1, PD-L1, and CD8 are shown in Fig. 1.

Positive expression of VISTA on TCs was observed more frequently in ER-negative, PR-negative, HER2-positive, triple-negative, high Ki67 proliferation index, and unfavorable molecular (HER2E and basal-like) subtypes (Supplementary Table 4). There was no association between the expression of VISTA on TCs and PD-1, PD-L1, CD8, or stromal TILs.

Table 2 Expression of VISTA on immune cells and immune markers in patients with invasive ductal carcinoma of the breast (validation cohort, $N = 578$)

Markers	*N* (%)	VISTA on immune cells		
		Negative	Positive	*p*-value
TILs				< 0.001
< 10%	354 (61.2)	308 (87.0)	46 (13.0)	
10–50%	187 (32.4)	102 (54.6)	85 (45.4)	
> 50%	37 (6.4)	7 (18.9)	30 (81.1)	< 0.001
CD8				
< 10%	433 (74.9)	368 (85.0)	65 (15.0)	
≥ 10%	145 (25.1)	49 (33.8)	96 (66.2)	< 0.001
PD-L1				
Negative	438 (75.8)	374 (85.4)	64 (14.6)	
Positive	140 (24.2)	43 (30.7)	97 (69.3)	
PD-1				< 0.001
Negative	461 (79.8)	387 (84.0)	74 (16.0)	
Positive	117 (20.2)	30 (25.6)	87 (74.4)	
VISTA on TCs				0.046
Negative	523 (90.5)	371 (70.9)	152 (29.1)	
Positive	55 (9.5)	46 (83.6)	9 (16.4)	

VISTA V-domain Ig suppressor of T-cell activation; *TIL* tumor-infiltrating lymphocyte; *PD-L1* programmed cell death-ligand 1; *PD-1* programmed cell death-1; *TC* tumor cell

Positive expression of VISTA correlates with a favorable prognosis in patients with IDC

After excluding patients with incomplete adjuvant systemic therapy or with follow-up times under six months, 513 patients (89%) were subjected to survival analysis. There were no significant differences between the validation cohort of 578 patients and the survival analysis cohort of 513 patients in terms of clinicopathological parameters and immune markers. The median follow-up period was 60 months (range, 6–73 months). During this period, 77 patients (15.0%) relapsed and 44 (8.6%) had died of breast cancer by January 2020.

As shown in Fig. 2 and Supplementary Table 5, positive expression of VISTA on ICs was significantly associated with improved RFS but was not associated with DSS in either the entire cohort or in HER2-positive patients. VISTA expression was significantly associated with improved RFS and DSS in patients with ER-negative, PR-negative, triple-negative, and basal-like subtypes. The Kaplan–Meier plotter analysis revealed that high C10orf54 mRNA expression was significantly associated with longer RFS in the entire cohort, PR-negative, luminal A, luminal B, HER2-enriched and basal-like subtypes (SupplementaryFig. 1 and 2).

On multivariate analyses, positive expression of VISTA was identified as an independent prognostic factor in terms of improved RFS and DSS in patients with ER-negative, PR-negative, and basal-like subtypes (Table 3). However, it was not an independent indicator of survival in the entire cohort or in patients with triple-negative, HER2-positive, or other intrinsic molecular subtypes after multivariate analyses of factors that included TILs, AJCC stage, tumor differentiation, and adjuvant therapy.

Discussion

VISTA is a novel immune checkpoint and a potential target for cancer immunotherapy. Although the importance of this protein in various cancers has previously been reported [9–13, 26–33], little is known about its expression in breast cancer. In the present study, we investigated VISTA expression and its association with other immune markers and clinicopathological parameters, as well as with ensuing clinical outcomes, in patients with IDC. VISTA was expressed on the stromal ICs and TCs of 29% and 8% of the patients, respectively. Expression of VISTA on ICs was associated with PD-1, PD-L1, CD8, stromal TILs, and unfavorable clinicopathological factors including ER-negativity, PR-negativity, HER2-positivity, triple-negativity, high Ki67 proliferation index, poor differentiation, HER2E subtype, and basal-like subtype. However, the expression of VISTA on ICs was associated with a favorable prognosis and was an

Table 3 Results of multivariate analyses for identifying survival-associated factors in patients with ER-negative ($N=179$), PR-negative ($N=209$), and basal-like ($N=99$) breast cancer

	Relapse-free survival		Disease-specific survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
ER-negative				
VISTA		0.010		0.025
Negative	1		1	
Positive	0.35 (0.16–0.78)		0.32 (0.12–0.87)	
AJCC stage		0.002[a]		0.004[a]
I	1		1	
II	4.10 (0.93–18.07)	0.062	4.68 (0.59–37.43)	0.146
III	9.68 (2.24–41.74)	0.002	14.23 (1.88–107.9)	0.010
PR-negative				
VISTA		0.014		0.027
Negative	1		1	
Positive	0.38(0.17–0.821)		0.33 (0.13–0.88)	
AJCC stage		0.001[a]		0.003[a]
I	1		1	
II	4.77 (1.09–20.87)	0.038	5.79 (0.73–45.68)	0.096
III	11.15 (2.60–47.83)	0.001	16.51(2.19–124.7)	0.007
Basal-like				
VISTA		0.032		0.044
Negative	1		1	
Positive	0.19 (0.04–0.87)		0.21 (0.05–0.96)	
AJCC stage		0.027[a]		0.047[a]
I	1		1	
II	5.23 (0.64–42.85)	0.123	4.53 (0.54–37.73)	0.163
III	12.42 (1.56–98.75)	0.017	10.68 (1.32–86.13)	0.026

ER estrogen receptor; *VISTA* V-domain Ig suppressor of T-cell activation; *PR* progesterone receptor; *AJCC* American Joint Committee on Cancer *HR* hazard ratio; *CI* confidence interval

[a]Overall significance as a prognostic factor

independent indicator of improved RFS and DSS in patients with ER-negative, PR-negative, and basal-like IDC.

Recent studies that investigated the role of VISTA expression in cancer progression and patients' clinical outcomes showed contradictory results. VISTA expression was found to be a poor prognostic factor in patients with early-stage head and neck squamous cell carcinoma and in those with primary cutaneous melanoma [12, 30]. In contrast, Loeser et al. found that VISTA correlated with improved OS in patients with pT1/2-stage esophageal adenocarcinoma [13], which was consistent with our findings in breast cancer. Moreover, recent studies demonstrated that VISTA was expressed in both the ICs and TCs of patients with gastric cancer, hepatocellular carcinoma, ovarian cancer, and lung cancer [9, 10, 29, 33]. In our current study, VISTA was expressed on the TCs of 8% of the patients but was not associated with survival. A previous study demonstrated that VISTA expression on TCs (but not ICs) was associated with significantly longer survival in patients with high-grade serous ovarian cancer, hepatocellular carcinoma, and lung cancer [9, 29, 33]. Recently, Mulati et al. found that VISTA expression in ovarian cancer cells suppressed T cell proliferation and cytokine production; moreover, VISTA decreased the number of tumor-infiltrating CD8-positive T cells [31]. However, the mechanisms of VISTA upregulation in TCs remain unknown. Taken together, these data suggest that the expression of VISTA on TCs and ICs may exert different functions and have distinct prognostic implications according to the type of cancer.

Although breast cancer is not generally immunogenic, its association with the immune system is well-documented based on the prognostic role of TILs [34]. Recent studies showed that some tumors, especially ER-negative breast cancers, elicit an immune response [15]. Our current study revealed that VISTA was enriched in ER-negative, PR-negative, HER2-positive, triple-negative, and unfavorable molecular subtype tumors (such as HER2E and basal-like subtypes), which is consistent with previous studies that found that the expression of PD-L1, lymphocyte activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) was associated with ER-negativity, PR-negativity, HER2-positivity, and unfavorable molecular subtypes [35–38]. Furthermore, Burugu et al. found both LAG-3-positive and TIM-3-positive TILs were independent favorable prognostic factors for ER-negative patients [35, 38], which is similar to our current findings concerning VISTA. These data suggest that the immune checkpoints on TILs are associated with clinical outcomes and may play vital roles in anti-tumor immunity.

The activation of alternative immune checkpoints could play a role in acquired resistance to immune checkpoint blockade [39]. Recent studies demonstrated that VISTA expression was elevated in patients with prostate cancer and melanoma after their treatment with CTLA4 or PD-1 blockers [6, 39], suggesting an important role for this protein in acquired resistance to immune checkpoint blockade. Therefore, combined VISTA and PD-1 blockade may produce a synergistic effect. Indeed, VISTA/PD-1 double knockout mice exhibited significantly increased immune-related events than did single knockout mice, suggesting that the immunoregulatory pathways for PD-1 and VISTA are functionally nonredundant during antigen-specific responses or autoimmune inflammatory conditions [7]. Moreover, the combined blockade of VISTA and PD-L1 using monoclonal antibodies led to a synergistic therapeutic effect, achieving

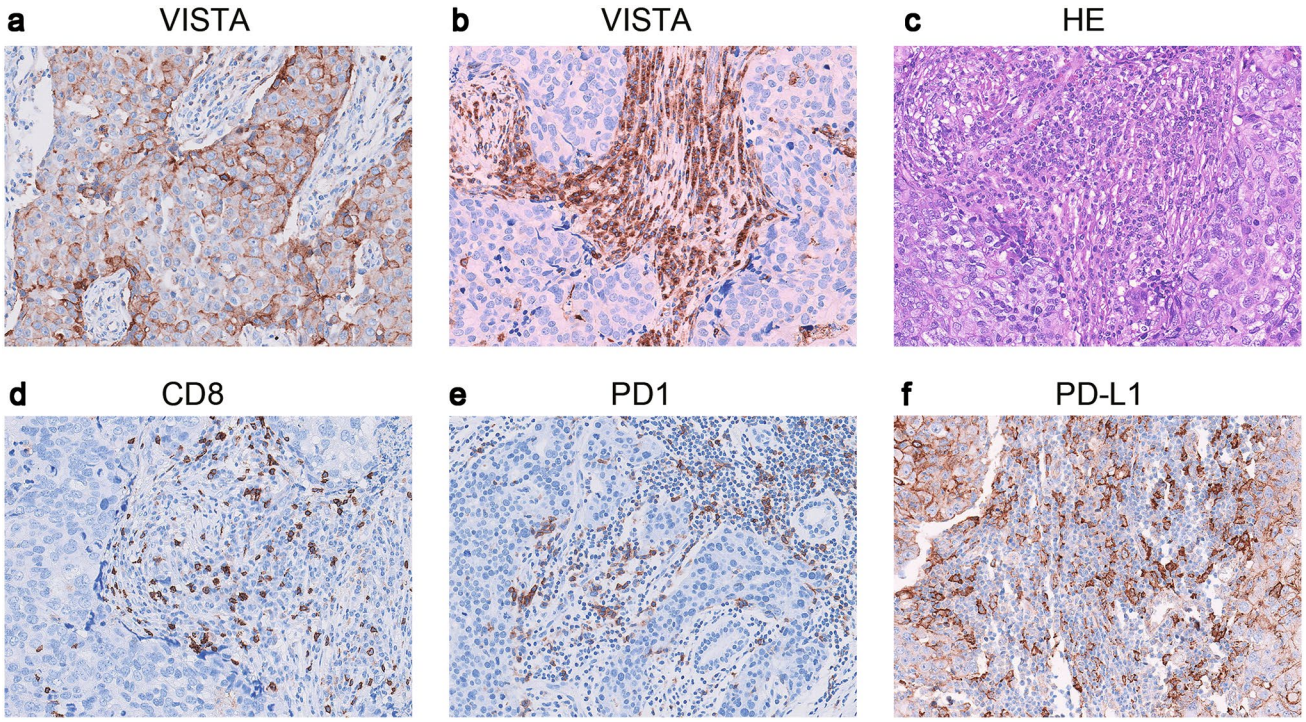

Fig. 1 Representative hematoxylin and eosin (HE) staining of tumor-infiltrating lymphocytes (TILs) and immunohistochemical staining of V-domain Ig suppressor of T-cell activation (VISTA), programmed cell death-1 (PD-1), PD-1 ligand-1 (PD-L1), and CD8 in human breast tumors. **a** VISTA expression on tumor cells; **b** VISTA expression on TILs; **c** HE staining of TILs; **d** CD8 expression on TILs; **e** PD-1 expression on TILs; **f** PD-L1 expression on TILs and tumor cells. Original magnifications × 200

optimal tumor suppression in a murine colon cancer model [7]. However, the association between VISTA and PD-1/PD-L1 pathways in breast cancer has not yet been fully elucidated. In the present study, we found a positive association between the levels of VISTA protein and PD-1/PD-L1, as well as complementary expression patterns for VISTA and PD-L1 in breast cancer specimens. This provided evidence that these proteins likely play a synergetic or cooperative role in breast cancer pathogenesis and immune evasion.

The blockade of VISTA alone or in combination with PD-1/PD-L1 represents a potentially promising strategy for human breast cancer immunotherapy. Future human clinical trials using VISTA inhibitor alone or combined with another immune checkpoint protein will be required to support this hypothesis.

Despite our novel findings, our study had some limitations including those inherent to a retrospective study. First, given the intra-tumoral heterogeneity, the use of TMAs may not have accurately represented the entire tumor insofar as marker expression. Second, it was difficult to assess the expression of VISTA in different subgroups of tumor-infiltrating ICs and to analyze the co-expression of VISTA and PD-L1 using immunohistochemistry. Future studies using multiplexed immunofluorescence methods are warranted to elucidate the expression and function of VISTA within the tumor-immune microenvironment. Furthermore, the sample size of our study was moderate and the number of events was relatively small, thereby compromising the statistical power of our subgroup analyses.

In conclusion, ours is the first investigation of VISTA expression in patients with breast cancer. We found that VISTA is associated with ER, PR, and HER2 expression; molecular subtypes; and PD-1/PD-L1 expression on TILs. Moreover, VISTA positivity was found to be an independent prognostic factor for improved RFS and DSS in patients with ER-negative, PR-negative, and basal-like IDC of the breast. These findings support VISTA as a target of therapeutic modulation alone or in combination with PD-1/PD-L1 blockade for human breast cancer immunotherapy.

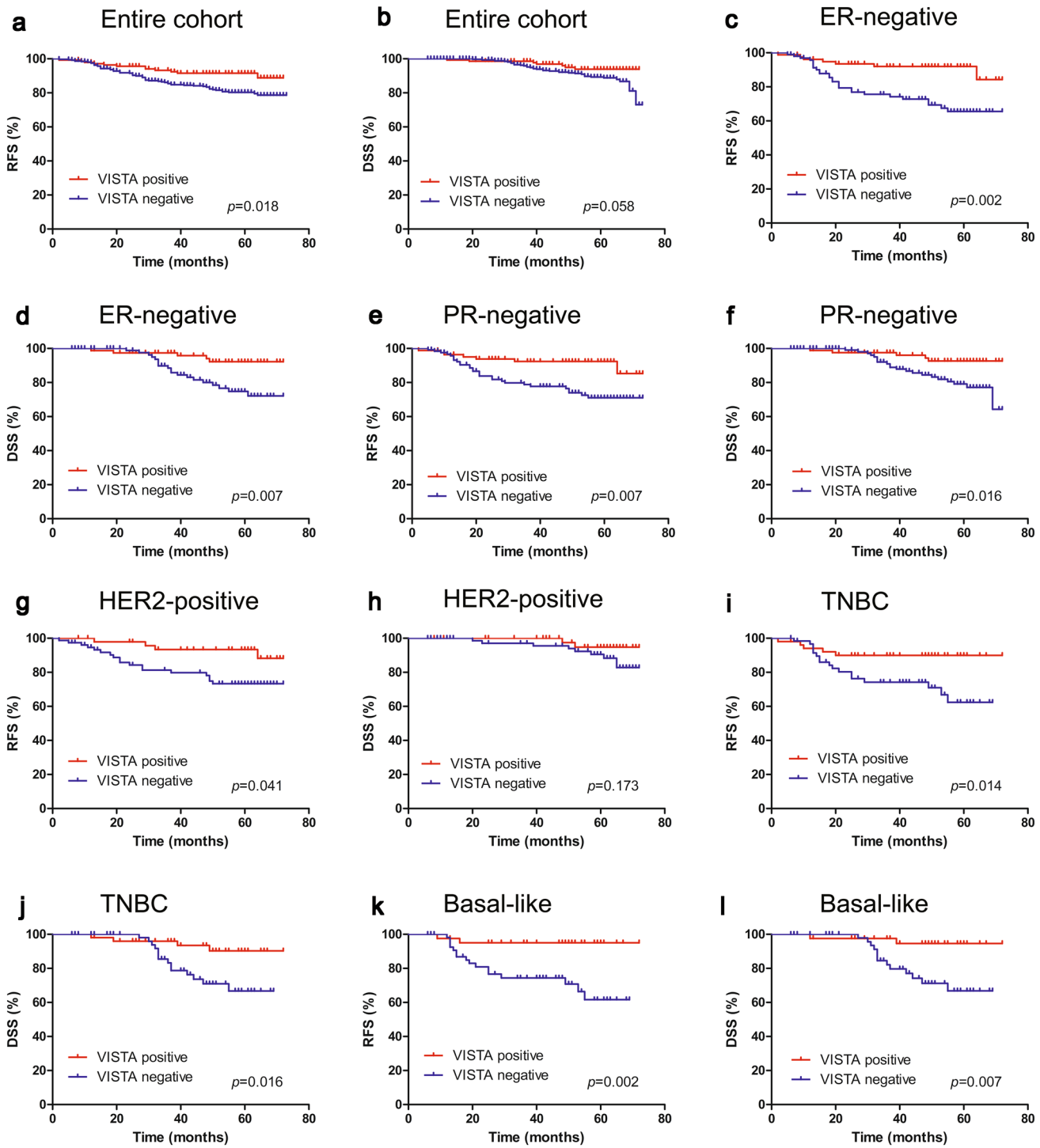

Fig. 2 Kaplan–Meier curves of relapse-free survival (RFS) and disease-specific survival (DSS) according to V-domain Ig suppressor of T-cell activation (VISTA) expression on immune cells in **a**, **b** the entire cohort, **c**, **d** estrogen receptor (ER)-negative patients, **e**, **f** progesterone receptor (PR)-negative patients, **g**, **h**) human epidermal growth factor receptor-2 (HER2)-positive patients, **i**, **j** patients with triple-negative breast cancer (TNBC), and **k**, **l** patients with basal-like morphology

Authors' contributions YX and JC made substantial contributions to the conception, design, and critical revision of the manuscript. LZ, SY, and SM made substantial contributions to tissue microarray construction, acquisition, and interpretation of the data, and drafting of the manuscript. YZ and MZ made substantial contributions to immunohistochemistry experiments and the interpretation of data. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval The study was approved by the Institutional Review Board of Peking Union Medical College Hospital (approval number S-K995).

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

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