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ARTICLE

Evaluation of glucocorticoid-induced TNF receptor (GITR) expression in breast cancer and across multiple tumor types

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Abstract

Glucocorticoid-induced TNF receptor (GITR) is an emerging immunotherapy target that is expressed at high levels on regulatory T cells. Agonistic anti-GITR antibodies have anti-tumor activity in cancer mouse models, and recent phase 1 trials have demonstrated their safe pharmacological profile. However, there is limited knowledge on the relationship between GITR expression and the tumor microenvironment. GITR protein expression was assayed by immunohistochemistry on 3992 breast cancer surgical excision specimens assembled into tissue microarrays and scored visually by a pathologist for GITR expression on tumor-infiltrating lymphocytes and on carcinoma cells. GITR expression by the malignant cells was further surveyed in gastrointestinal stromal tumor ($N = 713$), lung carcinoma ($N = 705$), pancreatic cancer ($N = 486$), ovarian cancer ($N = 445$), bladder cancer ($N = 88$), prostate cancer ($N = 88$), testicular cancer ($N = 76$), melanoma ($N = 75$), renal cell carcinoma ($N = 68$), epithelioid sarcoma ($N = 53$), and neuroendocrine tumors ($N = 41$). In breast cancer, GITR expression on tumor-infiltrating lymphocytes (12.4%) correlated with other immune response biomarkers (PD-L1+ on tumor cells, and PD-1+, LAG-3+, TIM-3+ lymphocytes; $p < 0.001$), and T-cell markers (CD8+, FOXP3+; $p < 0.001$). GITR+ carcinoma cells were observed in 6.0% of breast cancer cases and correlated with worse relapse-free survival ($p = 0.015$). Among the additional tumor types examined, cancers with GITR+ malignant cells included bladder cancer (5.7%), primary (but not metastatic) melanoma (4.5%), and ovarian cancer (3.2%); no expression was identified among examined sarcomas. To our knowledge, this is the first immunohistochemistry study to report the frequency and pattern of GITR expression in a large breast cancer cohort, or to report membranous GITR expression on malignant cells. The co-infiltration of GITR with other immune biomarkers and T-cell markers supports a potential role for anti-GITR agents in combination immunotherapies. In addition, GITR expression on carcinoma cells could imply the existence of a novel cancer immune evasion strategy worthy of further investigation.

Introduction

Immuno-oncology is one of the fastest growing fields in cancer research. By enhancing the patient's own immune system to target and eliminate tumor cells, immunotherapy aims to treat cancer without the toxic systemic effects of chemotherapy. The US Food and Drug Administration continues to approve new indications for the use of immune checkpoint inhibitors against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death receptor (PD-1) and its ligand (PD-L1) to treat cancers such as melanoma, lung cancer, and kidney cancer. While breast cancer was previously considered to be less immunogenic compared to, for example, melanoma and non-small cell lung cancer, studies show that HER2-positive and triple-negative breast cancer subtypes have increased tumor-

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infiltrating lymphocytes (TILs) [1], and that TILs are prognostic across breast cancer subtypes [2, 3]. Immune checkpoint blockade is currently the most investigated form of immunotherapy in breast cancer, and recent clinical trials show promising responses [4]. Among the plethora of novel immunotherapy monoclonal antibodies under investigation, glucocorticoid-induced tumor necrosis factor receptor (GITR) is an emerging target with the potential of being used in combination therapy with PD-1/PD-L1 inhibitors [5].

GITR is a transmembrane protein that acts as a co-stimulatory protein for T cells [6, 7]. It is constitutively expressed at high levels on regulatory T cells [8, 9] and at low levels on other immune cells such as conventional cytotoxic and helper T cells, natural killer cells, B cells and macrophages [10]. Upon binding to its ligand, GITRL, GITR increases proliferation of effector T cells [8, 11, 12] and dampens the suppressive activity of regulatory T cells [8, 13, 14]. Pre-clinical studies have demonstrated anti-tumor activity of agonistic anti-GITR antibodies in melanoma [15–17] and colorectal cancer mouse models [18]. Several human phase 1 trials of agonistic anti-GITR antibody monotherapy have reported its tolerable pharmacodynamic profile [19–22], supporting a viable role for targeting GITR in immunotherapy regimens.

RNA-seq analyses have shown the *GITR* gene to be upregulated in human whole breast tumors [23], and more specifically in regulatory T cells [24, 25]. However, no study in breast cancer has investigated GITR by immunohistochemistry (IHC), which assesses protein expression in its cellular and morphologic context. Our study is the first to report the distribution of GITR expression in a very large breast cancer cohort powered for association with major clinicopathological parameters and survival. Possibly even more importantly, we demonstrate the expression of GITR on carcinoma cells themselves, which to our knowledge has not been previously reported.

Materials and methods

Breast cancer study cohorts

An initial training cohort ($N = 330$ patients diagnosed with invasive breast cancer at the University of British Columbia hospital between 1989 and 2002, as previously described [26]) was used to finalize staining and interpretation conditions so these could be fully pre-specified and locked down before any application to the independent main study cohort. The main study cohort included 2499 patients yielding interpretable data, from an original series of 3992 patients diagnosed with invasive breast cancer who were referred to the British Columbia Cancer Agency between

Table 1 Association of GITR + intra-epithelial tumor-infiltrating lymphocytes (iTILs) with clinicopathological parameters and immune biomarkers in breast cancer.

Main study cohort ($N = 2499$)			
Parameters	Negative $n = 2190$	GITR + iTILs ≥1 $n = 309$ (12.4%)	p value
Age at diagnosis (years)			0.001
<50	624	116 (15.7%)	
≥50	1566	193 (11.0%)	
Grade			<0.001
1 or 2	990	80 (7.5%)	
3	1112	219 (16.5%)	
Unknown	88	10	
Nodal status			0.058
Negative	1193	187 (13.6%)	
Positive	991	122 (11.0%)	
Unknown	6	0	
ER			<0.001
Negative	555	154 (21.7%)	
Positive (>1%)	1633	154 (8.6%)	
Unknown	2	1	
PR			<0.001
Negative	961	201 (17.3%)	
Positive (>1%)	1116	96 (7.9%)	
Unknown	113	12	
Ki67 index			<0.001
Low	1147	97 (7.8%)	
High (≥ 14%)	877	197 (18.3%)	
Unknown	166	15	
Subtype			<0.001
Luminal A	921	66 (6.7%)	
Luminal B	635	88 (12.2%)	
HER2E	156	38 (19.6%)	
Basal-like	161	81 (33.5%)	
Triple negative, non-basal	139	25 (15.2%)	
Unassignable	178	11 (5.8%)	
PD-L1 (241/2918)			<0.001
Negative	1928	194 (9.1%)	
≥1%	117	98 (45.6%)	
PD-1 iTILs (246/2908)			<0.001
Negative	1963	187 (8.7%)	
≥1	117	108 (48.0%)	
LAG-3 iTILs (327/2921)			<0.001
Negative	1920	169 (8.1%)	
≥1	168	132 (44.0%)	
TIM-3 iTILs (332/2816)			<0.001
Negative	1912	201 (9.5%)	
≥1	184	99 (35.0%)	

Table 1 (continued)

Main study cohort ($N = 2499$)			
Parameters	Negative $n = 2190$	GITR + iTILs ≥1 $n = 309$ (12.4%)	p value
CD8+ **iTILs** (1089/ 3403)			<0.001
Negative	1455	105 (6.7%)	
≥1	627	195 (23.7%)	
FOXP3+ iTILs			<0.001
Negative	1483	78 (5.0%)	
≥2	607	219 (26.5%)	
% stromal TILs			<0.001
<10%	1733	169 (8.9%)	
≥10%	292	125 (30.0%)	
% stromal TILs (5% increments)			<0.001

1986 and 1996 from centers across the province, which has been previously described [27, 28]. Detailed clinicopathologic, treatment and outcome data were collected by the Breast Cancer Outcomes Unit of BC Cancer (see Table 1 for basic clinical and pathological parameters of the study population). For both breast cancer cohorts (initial training and main study), the median follow-up is 13 years, and no patient received neoadjuvant therapy. Samples and outcome data were de-identified and approved for access by the Clinical Research Ethics Board of the University of British Columbia.

IHC and scoring in breast cancer

Tissue microarrays with 0.6 mm cores were built using formalin-fixed, paraffin-embedded primary surgical excision specimens [27, 28]. Both breast cancer initial training and main study tumor tissue microarrays had been previously stained and scored by IHC for ER, PR, HER2, Ki67, CD5/6, CD8, FOXP3, LAG-3, PD-1, and PD-L1 [27, 29]. Hematoxylin and eosin-stained stromal TILs (H&E sTILs) counts were reported using the assessment recommendations from the International TILs Working Group [30] as previously described [29]. Breast cancer subtypes were defined using IHC benchmarked against a gene expression gold standard [31]. Specifically, Luminal A was defined as ER+ (≥1%) or PR+ (>20%), and HER2− and low Ki67 (<14%); Luminal B as ER+ or PR+ and any of: PR<20%, HER2+ or high Ki67 (>14%); HER2E as HER2+, ER and PR both negative; and basal-like as ER, PR and HER2 triple negative and EGFR+ or CK5/6+.

GITR IHC was performed using anti-human GITR rabbit monoclonal antibody D9I9D [32] (dilution 1:200; Cell Signaling, Danvers, MA, USA) on the Ventana Discovery Ultra automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Slides underwent 64 min of Cell Conditioning 1 (Ventana Medical Systems) for antigen retrieval, followed by 1 h of primary antibody incubation with no heat. Staining results were visualized using the UltraMap DAB anti-Rb Detection Kit (Ventana Medical Systems). Each run used membranous staining of tonsil tissue as a positive control. Lymphocytes with positive GITR expression were assessed by an experienced pathologist and reported in absolute visual counts per TMA core. Lymphocytes located within the carcinoma nests were defined as intra-epithelial TILs, and lymphocytes not in direct contact with the carcinoma nests were defined as stromal TILs. Carcinoma expression of GITR was reported as the percentage of malignant cells positive for membranous GITR expression.

To assess reproducibility, 250 cases were randomly selected, and were rescored by the study pathologist (DG) who scored the full series (blinded to her original interpretation) and were also scored by another breast cancer subspecialty pathologist (ZK) (Supplementary Table 1). The intra-observer concordance kappa value was 0.93 (95% CI: 0.91–0.94) for intra-epithelial TILs and 0.86 (95% CI: 0.83–0.89) for carcinoma cells. The inter-observer concordance kappa value was 0.51 (95% CI: 0.41–0.6) for intra-epithelial TILs and 0.81 (95% CI: 0.75–0.85) for carcinoma cells. To rule out cross-reactivity with another protein as the cause for the unexpected observation of membranous staining on cells other than lymphocytes, GITR expression on breast carcinoma cells was validated using an additional anti-human GITR rabbit monoclonal antibody recognizing a different epitope-binding site (Rabbit clone D5V7P, dilution 1:200 and 1:500, Cell Signaling). Primary images can be found on http://www.gpec.ubc.ca/gitr.php.

Additional study cohorts

A total of 11 additional types of human cancers were assessed on available tumor tissue microarrays to survey the prevalence of GITR expression on cancer cells themselves, according to the above-described assessment system established in breast cancer. Surgical specimens were obtained from hospitals in Vancouver, the University Hospital of North Norway, Columbia University, and the Fachklinik Hornheide at University Muenster. Five of these cohorts have been published previously: epithelioid sarcoma [33], gastrointestinal stromal tumors [34], melanoma [35], ovarian carcinoma [36], and pancreatic tumors [37].

Immunofluorescence

The cell lines BT-549 (breast cancer), HCT116 (colon cancer) and U2OS (osteosarcoma) were cultured in

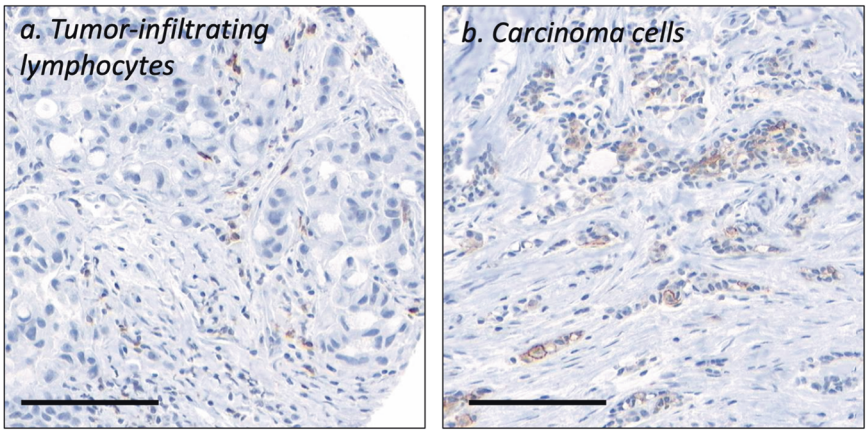

Fig. 1 GITR immunohistochemistry-stained breast cancer samples on tissue microarrays. **a** GITR membranous immunohistochemistry staining on tumor-infiltrating lymphocytes in the stroma and, in a minority of positive cells, in contact with carcinoma nests, and **b** GITR cytomembranous staining on breast carcinoma cells. Scale bars represent 100 µm.

RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA); PC3 cells (prostate cancer) were maintained in DMEM medium with 10% fetal bovine serum. All cells were cultured at 37 °C, 95% humidity and 5% CO_2. Approximately 25,000–50,000 cells of each cell line were seeded on each chamber of Millicell EZ 8-well glass slides (Millipore Sigma, Burlington, MA, USA) and grown overnight. The cells were fixed in 4% formaldehyde and permeabilized for 1 h with a blocking buffer containing 0.3% Triton X-100 and 5% bovine serum albumin. Cells were then incubated with rabbit anti-GITR antibody (D5V7P, Cell Signaling Technology; 1:200) or normal rabbit IgG of the same dilution overnight at 4 °C. Following incubation of fluorescent anti-rabbit Alexa594 secondary antibodies (Thermo Fisher Scientific) for 1 h, cells were then washed three times and mounted with Vectashield containing DAPI. Images were taken with a 65× oil lens at the Vancouver Prostate Center and processed with ZEISS ZEN 3.0.

Statistics

Statistical analyses were completed using IBM SPSS Statistics (version 25.0). GITR expression on iTILs was pre-specified, by analogy to previous studies of TIL biomarkers on these breast cancer tissue microarrays [29, 38, 39], to be dichotomized to positive (GITR+ iTIL ≥ 1) or negative (GITR+ iTIL = 0). Breast cancer-specific survival was the primary endpoint and was defined as the time from the date of diagnosis to the date of death attributed to breast cancer. Patients who were alive at the end of the follow-up or had other causes of death were censored. Relapse-free and overall survival were secondary endpoints. The former was defined as time of diagnosis to date of any breast cancer relapse (local, regional, distant, or contralateral), and the latter as time of diagnosis to date of death due to any cause. Univariate associations between GITR expression and clinicopathological parameters were analyzed by the Pearson's chi-square test. Kaplan–Meier analyses with log-rank test and Cox proportional hazard regression models were used to correlate GITR expression with survival. Subsequent multivariable Cox models adjusted for clinicopathological parameters (age, tumor grade and size, lymphovascular invasion, and nodal status), initial systemic therapy, and breast cancer subtype were built. The initial training cohort ($N = 330$) was analyzed first, the results of which were used to generate a pre-specified formal statistical plan for the main study cohort, which was presented to a meeting of the Breast Cancer Outcomes Unit. The main study cohort was further split in half for a training and validation approach when analyzing immune biomarker associations and prognostic correlations that were not pre-specified from the initial training cohort results (which had limited power and fewer available biomarker and clinical data fields). In these cases, a pre-specified statistical plan based on the first half of the main study cohort was presented prior to analyzing the second (validation) half. The statistical analyses for GITR expression on breast carcinoma cells were done only on the whole cohort as there were too few positive cases to allow a training and validation statistical approach. All statistical tests were two-sided at $\alpha = 0.05$.

Results

GITR expression on TILs in breast cancer

The initial cohort ($n = 330$) was used to optimize staining conditions and establish scoring criteria (Supplementary Table 2). The single core frequencies of stromal and intraepithelial TILs expressing GITR were comparable between the initial training and main study cohort ($n = 3992$). Of the 2499 (63%) interpretable cases in the main study cohort,

GITR+ stromal TILs were present in 853 cases (34%), and intra-epithelial TILs in 309 (12%) (Fig. 1).

For consistency with our previous studies on lymphocyte biomarker expression in breast cancer tissue microarrays [29, 38, 39], the presence of any GITR+ intra-epithelial TILs (≥1 per 0.6 mm tissue microarray core) was set as the cutoff to define a case as positive in correlational and survival analyses. GITR expression on breast cancer intra-epithelial lymphocytes was significantly associated with features of poor prognosis [40, 41] including younger age (<50 years), higher grade, ER and PR negativity, and high Ki67 proliferation index. Basal-like (34%) and HER2E (20%) subtypes were significantly enriched with GITR+ intra-epithelial TILs compared to luminal A (7%) and luminal B (10%) breast cancer subtypes. As expected, GITR expression on intra-epithelial TILs was positively associated with total lymphocyte infiltration as assessed by H&E sTILs (Table 1). Furthermore, cases with GITR+ intra-epithelial TILs were associated with concurrent infiltration of carcinoma cells expressing PD-L1, and with TILs expressing other immune checkpoint biomarkers (PD-1, LAG-3, TIM-3) and T-cell markers (CD8 and FOXP3). Expression of GITR on intra-epithelial TILs was not associated ($p > 0.05$) with overall, breast cancer-specific, or relapse-free survival in main study cohort (Supplementary Fig. 1) nor in exploratory analyses of major subtype strata (data not shown); multivariable analysis using the Cox regression model showed similarly nonsignificant results.

Concurrent tumor infiltration with GITR+ and FOXP3+ T cells

Regulatory T cells (CD4+/FOXP3+/CD25+) constitutively express GITR at high levels [13, 42], while effector (CD4+or CD8+) T cells express GITR in high levels when induced [7, 42]. Given the opposing roles of these two T cell populations in immune responses, GITR protein expression across all lymphocytes may not reflect the overall immune status of tumors. To address this, we analyzed concurrent tumor infiltration by GITR+ and FOXP3+ T cells in exploratory analyses. A higher proportion of ER negative tumors (39% of cases) had concurrent infiltration of FOXP3+ and GITR+ lymphocytes, compared to ER positive tumors (19% of cases) ($p = 0.002$). ER-negative tumors with concurrent GITR+ and FOXP3+ lymphocyte infiltration had improved breast-specific survival compared to tumors with a single or no positive T-regulatory marker TIL expression in univariate and multivariable analysis (Supplementary Fig. 2 and Supplementary Table 3). Neither FOXP3 (previously shown [27]) nor GITR alone (Supplementary Fig. 2) in breast cancer was significantly associated with clinical outcomes of patients with ER negative tumors.

Table 2 Association of GITR+ carcinoma cells with breast cancer clinicopathological parameters.

Main study cohort ($N = 2499$)			
Parameters	Negative $n = 2389$	GITR + carcinoma ≥1 $n = 150$ (6.0%)	p value
Grade			0.028
1 or 2	1018	51 (4.8%)	
3	1239	92 (6.9%)	
Unknown	91	7	
ER			0.048
Negative	676	32 (4.5%)	
Positive (>1%)	1669	118 (6.6%)	
Unknown	3	0	
PR			0.017
Negative	1104	57 (4.9%)	
Positive (>1%)	1124	88 (7.3%)	
Unknown	120	5	
Subtype			0.071
Luminal A	932	55 (5.6%)	
Luminal B	664	59 (8.2%)	
HER2E	185	8 (4.1%)	
Basal-like	231	11 (4.5%)	
Triple negative, non-basal	154	10 (6.1%)	
Unassignable	182	7 (3.7%)	
GITR+ iTILs (309/2190)			0.029
Negative	2049	140 (6.4%)	
≥1	299	10 (3.2%)	
PD-1 iTILs (246/2908)			0.085
Negative	2010	139 (6.5%)	
≥1	217	8 (3.6%)	
≥2	774	52 (6.3%)	
% stromal TILs			0.70
<10%	1782	119 (6.3%)	
≥10%	393	24 (5.8%)	
% stromal TILs (5% increments)			0.39

GITR expression on breast carcinoma cells

Unexpectedly, select breast cancer cases were observed to display membranous GITR expression on the carcinoma cells themselves, a finding which to our knowledge had not been reported in the past. Membranous staining of GITR on breast carcinoma cells was present on 150 cases (6%) in the main study cohort (Fig. 1), with the fraction of positive cells ranging from 5–95% on each TMA. Comparable positive carcinoma staining had also been observed in the initial training cohort. To confirm this finding, we repeated the IHC analysis using another anti-human GITR monoclonal

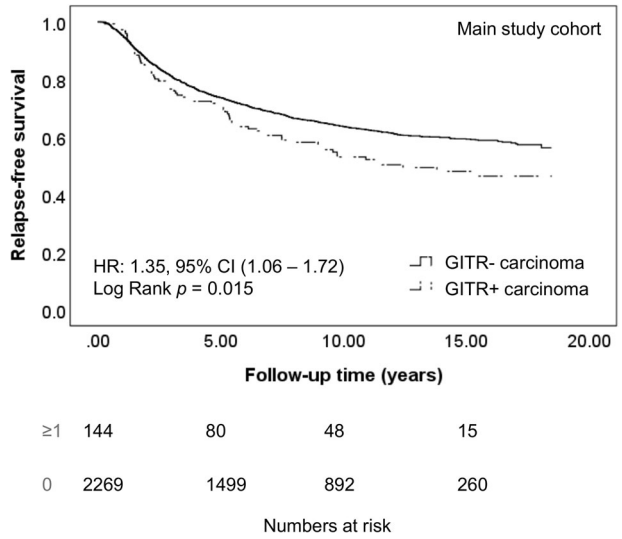

Fig. 2 Relapse-free survival stratified by GITR expression on breast carcinoma cells. Kaplan–Meier survival curves of relapse-free survival stratified by GITR expression on breast carcinoma cells in the main study cohort.

antibody that recognizes a different epitope as its binding site (Rabbit clone D5V7P, 1:200, Cell Signaling), and got the same results. GITR carcinoma expression, while rare, was somewhat more common in breast cancers of higher grade, ER and PR positivity, and luminal B subtype (Table 2). Breast tumors with GITR expression on carcinoma cells were less likely to have GITR expression on their intra-epithelial TILs. GITR expression on breast carcinoma cells was associated with worse relapse-free survival in the whole cohort in univariate (Fig. 2) and multivariate analyses (Table 3). There are 4 out of 148 (2.7%) cases with both GITR carcinoma expression and FOXP3 carcinoma nuclear staining (Supplementary Table 4).

GITR expression on malignant cells in additional tumor types

Since carcinoma cell expression of GITR had not been reported in the past, we proceeded to survey GITR expression on malignant cells by IHC in 11 additional tumor types. The frequency of membranous GITR expression on cancer cells differed by tumor type (Table 4). Breast (6.0%) and bladder cancer (5.7%) had the greatest fraction of positive cases, while others like melanoma, and carcinoma of renal cell, ovary, prostate, and lung expressed GITR less frequently (Fig. 3). The non-carcinomas tested (epithelioid sarcoma (Fig. 3), gastrointestinal stromal tumor, and testicular cancer) showed no GITR expression on cancer cells. Interestingly, there were 4.5% of primary melanoma specimens where malignant cells showed GITR expression, but no positive cases among melanoma specimens taken from metastatic sites.

Table 3 Multivariate analyses of GITR + carcinoma cells on the main study cohort for relapse-free survival (RFS).

Whole cohort (events = 884/2312)	Hazard ratio for RFS (95% CI)	*p* value
GITR+ carcinoma (ref. negative)		
Positive	1.32 (1.02–1.70)	0.036
Age at diagnosis (ref. <50)		
≥50	0.93 (0.77–1.12)	0.44
Grade (ref. Grade 1,2)		
Grade 3	1.35 (1.16–1.56)	<0.001
Nodal status (ref. negative)		
Positive	2.19 (1.82–2.65)	<0.001
LVI status (ref. negative)		
Positive	1.34 (1.14–1.57)	<0.001
Tumor size (ref. ≤2 cm)		
>2 cm	1.39 (1.20–1.59)	<0.001
Initial systemic therapy (ref. no systemic therapy)		<0.001
Tamoxifen only	0.62 (0.50–0.77)	<0.001
Chemotherapy only	0.65 (0.51–0.84)	0.001
Chemo + Tamoxifen	0.63 (0.47–0.86)	0.004
Breast cancer subtype (ref. luminal A)		<0.001
Luminal B	1.44 (1.22–1.70)	<0.001
HER2E	1.64 (1.28–2.10)	<0.001
Basal-like	1.30 (1.02–1.67)	0.034
Triple negative, not basal	0.99 (0.72–1.36)	0.95
Unassignable	1.14 (0.86–1.50)	0.36

Table 4 Frequency of GITR expression on carcinoma cells among different tumor microarrays.

Tumor type	Total	Interpretable	GITR+ carcinoma
Breast cancer	3992	2499	150 (6.0%)
Bladder cancer	88	87	5 (5.7%)
Melanoma (primary)	75	66	3 (4.5%)
Ovarian cancer	445	431	14 (3.2%)
Renal cell carcinoma	68	64	2 (3.1%)
Neuroendocrine tumor	41	40	1 (2.5%)
Prostate cancer	88	88	2 (2.3%)
Lung carcinoma	705	662	7 (1.0%)
Pancreatic cancer	486	444	4 (0.9%)
Epithelioid sarcoma	53	37	0 (0%)
Gastrointestinal stromal tumor	713	647	0 (0%)
Melanoma (metastases)	65	56	0 (0%)
Testicular cancer	76	55	0 (0%)

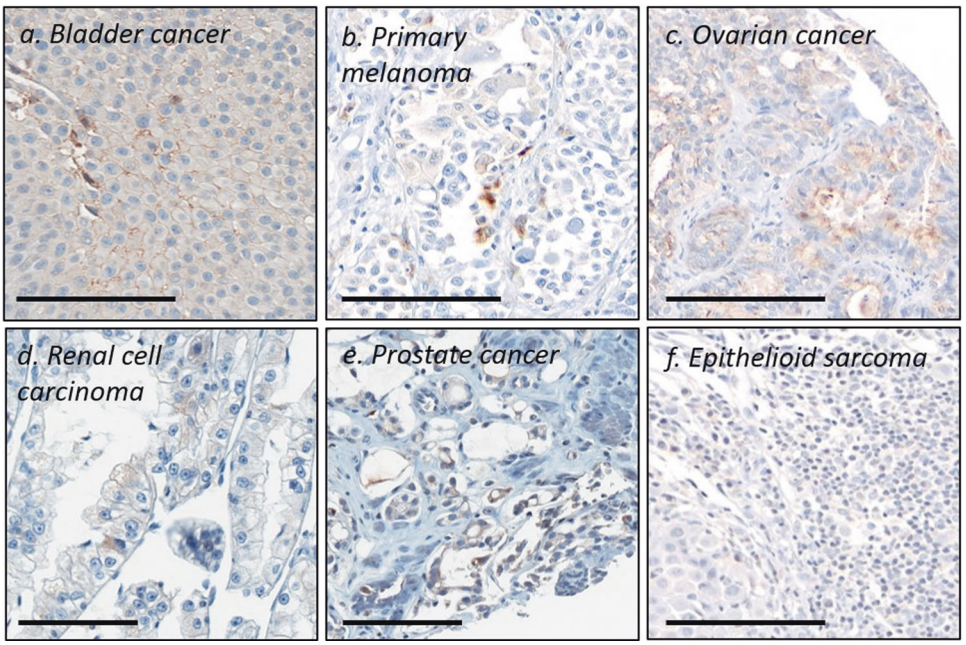

Fig. 3 Positive GITR cytomembranous staining on primary carcinoma cells. Positive GITR cytomembranous staining on primary carcinoma cells of **a** bladder, **b** melanoma, **c** ovary, **d** clear cell, and **e** prostate. **f** Negative GITR staining on malignant cells of epithelioid sarcoma. Scale bars represent 100 µm.

To confirm the observations from IHC using a more sensitive and precise technique on independent materials, immunofluorescence was used to image the localization of GITR protein expression. Using the Broad Institute Cancer Cell Line Encyclopedia database [43], we identified two cell lines with high expression of *GITR* at the RNA level (PC3 prostate carcinoma and HCT116 colorectal carcinoma) and two cell lines with much lower *GITR* expression (BT-549 triple negative breast cancer and U2OS osteosarcoma). Immunofluorescence images confirmed membranous expression of GITR protein on PC3 and HCT116 cells, whereas none was observed on BT-549 and U2OS cells (Fig. 4).

Discussion

This is the first study in breast cancer to report GITR expression on both TILs and carcinoma cells using IHC. We report that 34% of patients had GITR+ lymphocytes in the stromal compartment and 12% in the intra-epithelial compartment on standard 0.6 mm tissue microarray core samples from surgical specimens. GITR expression in lymphocytes is associated with younger age, higher tumor grade, ER and PR positivity, and immune checkpoint biomarkers (PD-1, LAG-3, TIM-3, PD-L1) and T-cell markers (CD8 and FOXP3). Furthermore, novel GITR expression on cancer cells themselves was confirmed in a minority of cases of carcinomas of the breast, bladder, renal cell, ovary, neuroendocrine, prostate, and lung, as well as melanoma. Carcinoma cell expression of GITR on breast cancer was associated with worse relapse-free survival.

GITR on TILs in breast cancer

We report that GITR correlates with immune checkpoint biomarkers (PD-1, LAG-3, TIM-3, PD-L1) and T-cell markers (CD8 and FOXP3). Consistent with our findings, using IHC on thymic epithelial tumors Arbour et al. [44] showed that high GITR expression on TILs was associated with co-expression of PD-1. However, they did not find co-association of GITR expression with TIM-3 or PD-L1 [44]. The discrepancy suggests that correlation between GITR and specific immune checkpoints may be tumor-specific. In a single-cell RNA-seq study of immune cells in breast carcinoma, Azizi et al. [45] reported strong covariation between GITR and CTLA-4 in certain regulatory T cell clusters but not others. Their study agrees with our observation, in which concurrent infiltration of GITR and other immune biomarkers was present in some but not all cases, suggesting that the mechanism for selecting GITR expression may be different but related to mechanisms inducing other immune checkpoints.

As expected given the biological role of GITR in T cells, our results show a correlation, in breast cancer, between the presence of lymphocytes expressing GITR and those expressing T-cell markers (CD8 and FOXP3). Similar correlations were reported by Vence et al. [46] in five different carcinomas. Fundamentally, the association between GITR, other immune checkpoints, and T-cell markers observed in our study supports the argument that GITR agonists may

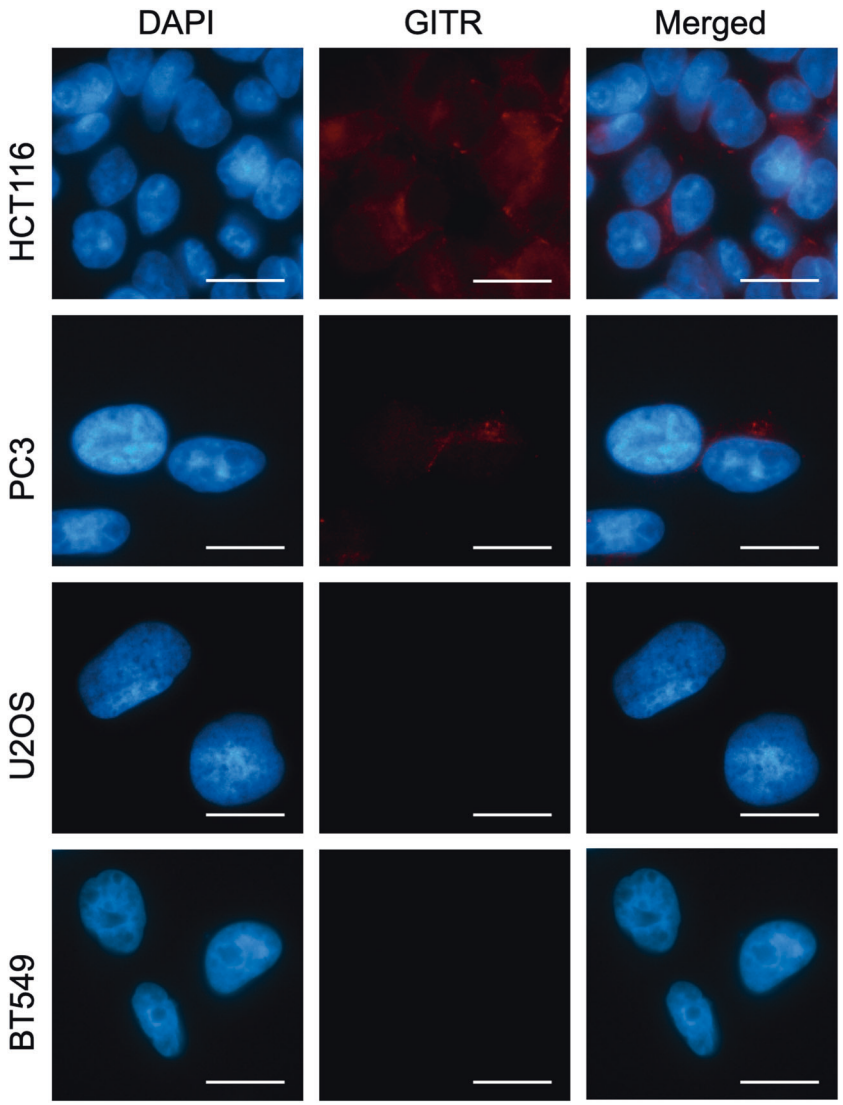

Fig. 4 Immunofluorescence staining of cancer cell lines (HCT116, PC3, U2OS, and BT549). The cells were stained with DAPI (nuclei, blue), and anti-GITR antibody (red). Scale bars represent 20 μm.

find a role in combination therapies, as proposed by Zappasodi et al. [22].

The concurrent infiltration of FOXP3+ and GITR+ TILs was associated with improved clinical outcomes in ER-negative breast cancer. These results suggest that FOXP3 and GITR together are more representative of the immune status of ER-negative breast tumors compared to individual markers. This may be indicative of a more active tumor-infiltrating regulatory T-cell population in non-luminal breast cancers, since FOXP3 overexpression induces GITR expression in regulatory T cells [47–49]. Furthermore, GITR expression alone was not prognostic in breast cancer, likely because GITR can be induced to high level expression in both conventional and regulatory T cells. The opposing roles of these T-cell populations in tumor immunity could impede the use of GITR as a single prognostic marker. Echoing recent findings on the benefits of using multiplex methodologies [50], our results suggest that GITR may be more informative when used in combination with other T-cell markers.

GITR+ carcinoma

To our knowledge, GITR protein expression on carcinoma cells is a novel finding. Past studies have only reported GITR expression on immune cells including T cells, myeloid cells, and neutrophils. While previous study [51] and public gene expression datasets [43] have reported non-zero value of *GITR* mRNA in cancer cell lines, we are the first study to survey the frequency of GITR protein expression across a large number of different cancer specimens. Interestingly, GITR expression on the neoplastic cells themselves was restricted to carcinomas/melanomas and not the examined sarcomas or germ cell tumors. In keeping with reports that carcinoma cells can express PD-L1 [52], it is those epithelial cell malignancies with relatively high

immunogenicity, such as melanoma and bladder cancer, that have higher frequencies of cases with GITR+ neoplastic cells. However, GITR expression on the neoplastic cells was not associated with stromal TIL infiltration, and was negatively correlated with GITR+ TILs.

In addition, GITR carcinoma cell expression in breast cancer was associated with worse prognosis. In conjunction with the observation that breast cancer had the highest proportion of GITR+ carcinoma cases compared to the other examined tumor types, our findings suggest that GITR carcinoma expression may have potential implications for breast cancer immunotherapy. Interestingly, past studies have reported FOXP3 carcinoma expression to also be the highest in breast cancer compared to other tumor types such as bladder cancer, melanoma, and non-small cell lung cancer [53]. There may be similar mechanisms leading to carcinoma cells expressing both GITR and FOXP3, given their close association in regulatory T-cell activity [54].

Moreover, a past study has reported carcinoma cell expression of GITR ligand (GITRL) in multiple carcinoma cell lines including HCT116 colon, 2102Ep embryonal, and MCF7 breast [55]. The GITRL+ carcinoma cells were demonstrated to confer immunosuppressive activity after activating GITR on natural killer cells [55]. Our findings suggest that tumors may also activate an alternative immuno-evasion strategy through the binding of GITR on carcinoma cells with GITRL expressed on other cell types. Tumor expression of FOXP3 was shown to have both direct and indirect suppressive activity on the proliferation of activated T cells [56, 57], and a similar mechanism may hold true for tumor expression of GITR. Alternatively, it is possible that GITRL availability determines the frequency and composition of GITR+ cells in the tumor microenvironment. The negative association between GITR+ TILs and GITR+ breast carcinoma cells in our results could be due to ligand-binding competition between GITR+ TILs and breast carcinoma cells. Given that GITR signaling on TILs is known to promote cell survival and proliferation, ligands bound to GITR on breast cancer cells may become unavailable to TILs and could lead to reduced anti-tumor activity of GITR.

Strengths and limitations

Strengths of this study include the incorporation of a particularly large cohort of breast cancer patients treated using standardized provincial guidelines and associated with long-term outcome data, and the use of an initial training and independent main study cohort to minimize type I error during analysis. The novel finding of GITR expression on carcinoma cells was supported by surveying 11 additional types of tumor, by applying two antibodies targeting different GITR epitopes, and by using two different visualization techniques. The limitations of this study are predominantly due to the methodology chosen. While tumor microarrays can screen a large cohort efficiently, the 0.6 mm cores used to build the tumor microarrays will not reliably detect biomarkers expressed in focal areas of a tumor. In addition, given the usage of a single immunohistochemical stain at a time, we could only infer the concurrent infiltration of immune cells in a particular core through analysis of serial sections, and could not directly visualize co-expression of immune biomarkers on individual cells. Assessing GITR expression by IHC and immunofluorescence may not directly reflect its activation status. In addition, the size of non-breast cancer cohorts available to us was insufficient to power clinical correlative analyses of GITR expression on malignant cells, an event that is relatively rarely detected on TMA core samples.

Conclusion and future directions

GITR is detectable in a subset of breast cancer tumor specimens, both on lymphocytes and carcinoma cells. The expression of GITR on carcinoma cells is a novel and potentially important aspect about GITR that is worthy of further investigation, to help advance immunotherapy strategies that target GITR. Future investigations should focus on elucidating the function of GITR on carcinoma cells and understanding its mechanism in anti-tumor immunity. In addition, concurrent infiltration of GITR with other immune biomarkers suggests a potential role for GITR in combination therapy, an area currently under investigation in multiple clinical trials (NCT02697591, NCT02740270, NCT03126110, NCT03277352, NCT03707457).

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Compliance with ethical standards

Conflict of interest TON has a proprietary interest (Bioclassifer LLC, Nanostring Technologies) in the PAM50 subtype classifier, not used in this study. The rest of the authors declare no conflict of interest.

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References

1. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: From TILs to the clinic. Nat Rev Clin Oncol. 2016;13:228–41.
2. Pusztai L, Karn T, Safonov A, Abu-Khalaf MM, Bianchini G. New strategies in breast cancer: Immunotherapy. Clin Cancer Res. 2016;22:2105–10.
3. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19:40–50.
4. Adams S, Gatti-Mays ME, Kalinsky K, Korde LA, Sharon E, Amiri-Kordestani L, et al. Current landscape of immunotherapy in breast cancer: a review. JAMA Oncol. 2019;5:1205–14.
5. Burugu S, Dancsok AR, Nielsen TO. Emerging targets in cancer immunotherapy. Semin Cancer Biol. 2018;52:39–52.
6. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol. 2013;13:227–42.
7. Clouthier DL, Watts TH. Cell-specific and context-dependent effects of GITR in cancer, autoimmunity, and infection. Cytokine Growth Factor Rev. 2014;25:91–106.
8. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol. 2002;3:135–42.
9. Coe D, Begom S, Addey C, White M, Dyson J, Chai J-G. Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy. Cancer Immunol Immunother. 2010;59:1367–77.
10. Krausz LT, Bianchini R, Ronchetti S, Fettucciari K, Nocentini G, Riccardi C. GITR-GITRL system, a novel player in shock and inflammation. Sci World J. 2007;7:533–66.
11. Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, et al. Frontline: GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. Eur J Immunol. 2004;34:613–22.
12. Shevach EM, Stephens GL. Opinion: The GITR-GITRL interaction: Co-stimulation or contrasuppression of regulatory activity? Nat Rev Immunol. 2006;6:613–8.
13. McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, et al. CD4+CD25+ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity. 2002;16:311–23.
14. Ji H, Liao G, Faubion WA, Abadia-Molina AC, Cozzo C, Laroux FS, et al. Cutting Edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. J Immunol. 2004;172:5823–7.
15. Ramirez-Montagut T, Chow A, Hirschhorn-Cymerman D, Terwey TH, Kochman AA, Lu S, et al. Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. J Immunol. 2006;176:6434–42.
16. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3 + CD25 + CD4 + regulatory T cells. J Exp Med. 2012;209:423–423.
17. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med. 2013;210:1389–402.
18. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, et al. Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med. 2013;210:1685–93.
19. Koon HB, Shepard DR, Merghoub T, Schaer DA, Sirard CA, Wolchok JD. First-in-human phase 1 single-dose study of TRX-518, an anti-human glucocorticoid-induced tumor necrosis factor receptor (GITR) monoclonal antibody in adults with advanced solid tumors. J Clin Oncol. 2018;34:3017.
20. Geva R, Voskoboynik M, Beebe AM, Gwo J, Dobrenkov K, Chartash E, et al. First-in-human phase 1 study of MK-1248, an anti-human glucocorticoid-induced tumor necrosis factor receptor (GITR) monoclonal antibody, as monotherapy or in combination with pembrolizumab in patients with advanced solid tumors. J Clin Oncol. 2018;36:3029.
21. Tran B, Carvajal RD, Marabelle A, Patel SP, Lorusso PM, Rasmussen E, et al. Dose escalation results from a first-in-human, phase 1 study of glucocorticoid-induced TNF receptor-related protein agonist AMG 228 in patients with advanced solid tumors. J Immunother Cancer. 2018;6:1–9.
22. Zappasodi R, Sirard C, Li Y, Budhu S, Abu-Akeel M, Liu C, et al. Rational design of anti-GITR-based combination immunotherapy. Nat Med. 2019;25:759–66.
23. Cari L, Nocentini G, Migliorati G, Riccardi C. Potential effect of tumor-specific Treg-targeted antibodies in the treatment of human cancers: a bioinformatics analysis. Oncoimmunology. 2018;7:e1387705.
24. De Simone M, Arrigoni A, Rossetti G, Gruarin P, Ranzani V, Politano C, et al. Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells. Immunity. 2016;45:1135–47.
25. Plitas G, Konopacki C, Wu K, Bos PD, Morrow M, Putintseva EV, et al. Regulatory T cells exhibit distinct features in human breast cancer. Immunity. 2016;45:1122–34.
26. Bortnik S, Choutka C, Horlings HM, Leung S, Baker JHE, Lebovitz C, et al. Identification of breast cancer cell subtypes sensitive to ATG4B inhibition. Oncotarget. 2016;7:66970–88.
27. Liu S, Foulkes WD, Leung S, Gao D, Lau S, Kos Z, et al. Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. Breast Cancer Res. 2014;16:1–12.
28. Cheang MCU, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res. 2008;14:1368–76.
29. Burugu S, Gao D, Leung S, Chia SK, Nielsen TO. TIM-3 expression in breast cancer. Oncoimmunology. 2018;7:1–9.
30. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: recommendations by an International TILS Working Group 2014. Ann Oncol. 2015;26:259–71.
31. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;101:736–50.
32. Shibutani M, Maeda K, Nagahara H, Fukuoka T, Matsutani S, Kashiwagi S, et al. A comparison of the local immune status between the primary and metastatic tumor in colorectal cancer: a retrospective study. BMC Cancer. 2018;18:1–4.
33. Pacheco M, Nielsen TO. Histone deacetylase 1 and 2 in mesenchymal tumors. Mod Pathol. 2012;25:222–30.
34. Steigen SE, Straume B, Turbin D, Chan AKW, Leung S, Nielsen TO, et al. Clinicopathologic factors and nuclear morphometry as independent prognosticators in KIT-positive gastrointestinal stromal tumors. J Histochem Cytochem. 2008;56:139–45.

35. Perez-Lorenzo R, Gill KZ, Shen CH, Zhao FX, Zheng B, Schulze HJ, et al. A tumor suppressor function for the lipid phosphatase INPP4B in melanocytic neoplasms. J Investig Dermatol. 2014;134:1359–68.
36. Kalloger SE, Köbel M, Leung S, Mehl E, Gao D, Marcon KM, et al. Calculator for ovarian carcinoma subtype prediction. Mod Pathol. 2011;24:512–21.
37. Kalloger SE, Riazy M, Tessier-Cloutier B, Karasinska JM, Gao D, Peixoto RD, et al. A predictive analysis of the SP120 and 10D7G2 antibodies for human equilibrative nucleoside transporter 1 (hENT1) in pancreatic ductal adenocarcinoma treated with adjuvant gemcitabine. J Pathol Clin Res. 2017;3:179–90.
38. Burugu S, Gao D, Leung S, Chia SK, Nielsen TO. LAG-3+ tumor infiltrating lymphocytes in breast cancer: clinical correlates and association with PD-1/PD-L1+ tumors. Ann Oncol. 2017;28:2977–84.
39. Liu S, Lachapelle J, Leung S, Gao D, Foulkes WD, Nielsen TO. CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. Breast Cancer Res. 2012;14:R48.
40. Fitzgibbons PL, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, et al. Prognostic factors in breast cancer. Arch Pathol Lab Med. 2000;124:966–78.
41. Cianfrocca M, Goldstein LJ. Prognostic and predctive factors in early-stage breast cancer. Oncologist. 2004;9:606–16.
42. Krausz LT, Bianchini R, Ronchetti S, Fettucciari K, Nocentini G, Riccardi C. GITR-GITRL System, a Novel Player in Shock and Inflammation. Sci World J. 2007;7:533–66.
43. Ghandi M, Huang FW, Jané-Valbuena J, Kryukov GV, Lo CC, McDonald ER, et al. Next-generation characterization of the Cancer Cell Line Encyclopedia. Nature. 2019;569:503–8.
44. Arbour KC, Naidoo J, Steele KE, Ni A, Moreira AL, Rekhtman N, et al. Expression of PD-L1 and other immunotherapeutic targets in thymic epithelial tumors. PLoS One. 2017;12:1–11.
45. Azizi E, Carr AJ, Plitas G, Cornish AE, Konopacki C, Prabhakaran S, et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. Cell. 2018; 174:1293–1308.e36.
46. Vence L, Bucktrout SL, Fernandez Curbelo I, Blando J, Smith BM, Mahne AE, et al. Characterization and comparison of GITR expression in solid tumors. Clin Cancer Res. 2019; clincanres.0289.2019.
47. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057–61.
48. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+T regulatory cells. Nat Immunol. 2003;4:337–42.
49. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4:330–6.
50. Lu S, Stein JE, Rimm DL, Wang DW, Bell JM, Johnson DB, et al. Comparison of biomarker modalities for predicting response to PD-1/PD-L1 checkpoint blockade. JAMA Oncol. 2019;21287:1195–204.
51. Kwon B, Yu K-Y, Ni J, Yu G-L, Jang I-K, Kim Y-J, et al. Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. J Biol Chem. 1999;274:6056–61.
52. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med. 2002;8:793–800.
53. Triulzi T, Tagliabue E, Balsari A, Casalini P. FOXP3 expression in tumor cells and implications for cancer progression. J Cell Physiol. 2013;228:30–35.
54. Knee DA, Hewes B, Brogdon JL. Rationale for anti-GITR cancer immunotherapy. Eur J Cancer. 2016;67:1–10.
55. Baltz KM, Krusch M, Bringmann A, Brossart P, Mayer F, Kloss M, et al. Cancer immunoediting by GITR (glucocorticoid-induced TNF-related protein) ligand in humans: NK cell/tumor cell interactions. FASEB J. 2007;21:2442–54.
56. Niu J, Jiang C, Li C, Liu L, Li K, Jian Z, et al. Foxp3 expression in melanoma cells as a possible mechanism of resistance to immune destruction. Cancer Immunol Immunother. 2011;60:1109–18.
57. Hinz S, Pagerols-Raluy L, Oberg HH, Ammerpohl O, Grüssel S, Sipos B, et al. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res. 2007;67:8344–50.