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Original Article

Transcriptomic analysis of GITR and GITR ligand reveals cancer immune heterogeneity with implications for GITR targeting

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Abstract: Glucocorticoid-induced tumor necrosis factor related protein (GITR) is a transmembrane protein expressed mostly on $CD25^+CD4^+$ regulatory T-cells (Tregs) and upregulated on all T-cells upon activation. It is a T-cell co-stimulatory receptor and has demonstrated promising anti-tumor activity in pre-clinical studies. To date, however, the efficacy of GITR agonism has been discouraging in clinical trials. This study explores GITR and GITR ligand (GITR-L) ribonucleic acid (RNA) expression in solid tumors in an attempt to delineate causes for variable responses to GITR agonists. RNA expression levels of 514 patients with a variety of cancer types were normalized to internal housekeeping gene profiles and ranked as percentiles. 99/514 patients (19.3%) had high GITR expression (defined as ≥ 75th percentile). Breast and lung cancer had the highest proportion of patients with high GITR expression (39% and 35%, respectively). The expression of concomitant high GITR and low-moderate GITR-L expression (defined as <75th percentile) was present in 31% and 30% of patients with breast and lung cancer respectively. High GITR expression also showed a significant independent association with high RNA expression of other immune modulator proteins, namely, PD-L1 immunohistochemistry (IHC) ≥1 (odds ratio (OR) 2.15, P=0.008), CTLA4 (OR=2.17, P=0.05) and OX40 high RNA expression (OR=2.64, P=0.001). Overall, these results suggest that breast and lung cancer have a high proportion of patients with a GITR and GITR-L RNA expression profile that merits further investigation in GITR agonism studies. The association of high GITR expression with high CTLA4 and OX40 RNA expression, as well as positive PD-L1 IHC, provides a rationale for a combination approach targeting these specific immune modulator proteins in patients whose tumors show such co-expression.

Keywords: Immunotherapy, GITR, precision oncology, biomarkers, transcriptomics

Introduction

Glucocorticoid-induced tumor necrosis factor related protein (GITR), also known as tumor necrosis factor (TNF) receptor superfamily member 18 (TNFRSF18) or cluster of differentiation (CD) 357 was initially defined as a glucocorticoid receptor belonging to the TNF superfamily as early as 1997 [1]. It is expressed at high levels on T-regulatory (Treg) cells, and its expression is also upregulated on activated natural killer (NK) and effector $CD4^+$ and $CD8^+$ T-cells (including tumor infiltrating lymphocytes (TILs)) relative to their naive counterparts [2, 3]. It is also expressed at low levels on B-cells [2]. GITR's main ligand, glucocorticoid-induced tumor necrosis factor related protein ligand (GITR-L), or tumor necrosis factor superfamily member 18 (TNFSF18), is mainly expressed on antigen-presenting cells, namely, dendritic cells, B-cells and monocytes, but also on endothelial cells [2]. The interaction of GITR with its

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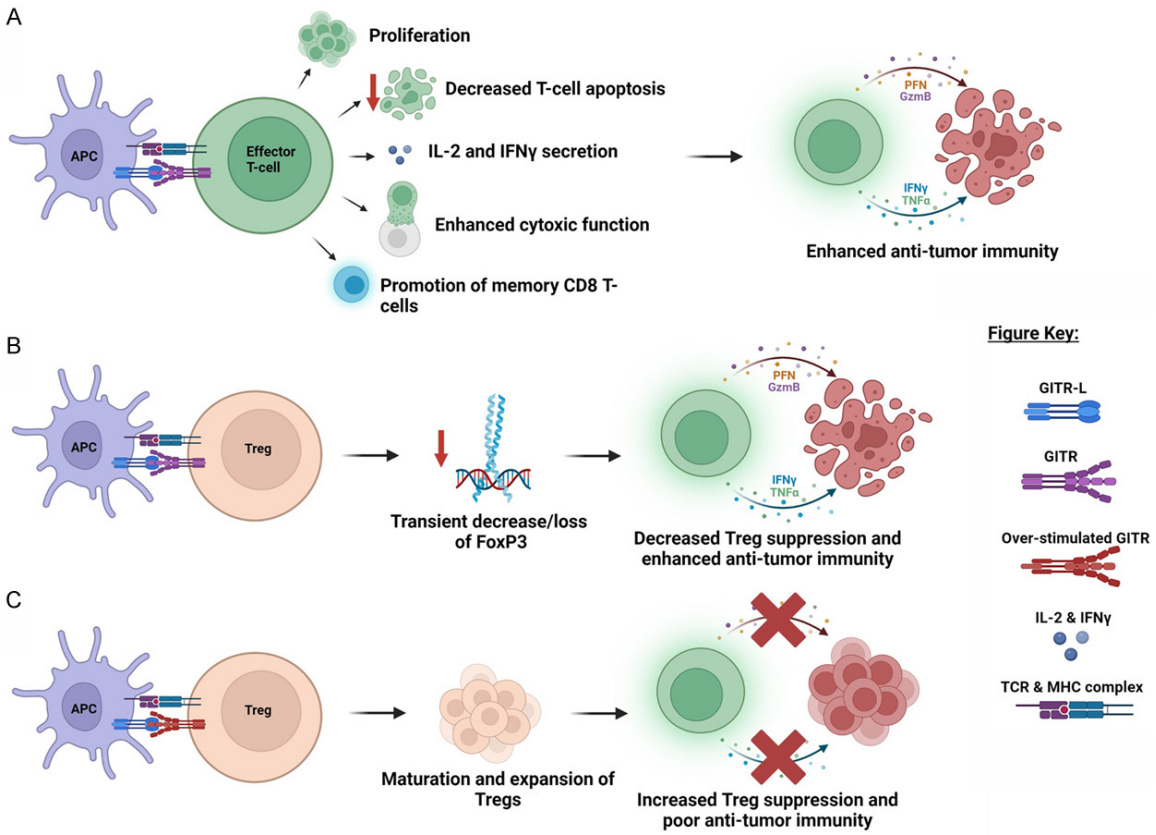

Figure 1. Simplified mechanistic depiction illustrating the effects of GITR and GITR-L interaction (once co-stimulated with the primary signal from TCR and MHCII) on the immune system and anti-tumor immunity. (A) The downstream co-activating effects of GITR triggering on effector T-cells leads to increased IL-2 and IFN-γ secretion and enhances proliferation/protects from apoptosis (via BCL-xL up-regulation) as well as augments cytotoxic function and promotion of memory effector cells, leading to enhanced anti-tumor immunity. (B) The downstream effects of short-term GITR triggering on Treg cells are shown. These effects lead to dampened FoxP3 expression, ultimately resulting in a decrease in Treg suppression of effector T-cells and enhanced anti-tumor immunity. (C) Long-term or over-stimulated GITR in Tregs, leads to an opposite effect of that illustrated in (B), namely, maturation and expansion of Tregs, which increases Treg suppression of effector T cells and attenuates anti-tumor immunity.

ligand leads to GITR activation and signal transduction mainly through the nuclear factor kappa B (NF-κB) pathway. On activated effector T-cells, pre-clinical murine studies have shown that GITR agonism via GITR-L or antibodies leads to a decrease in T cell apoptosis (via B-cell lymphoma-extra large (BCL-xL) up-regulation), as well as potentiation of T cell activation by upregulating CD25 expression via induction of interleukin 2 (IL-2) and interferon gamma (IFNγ) expression, all of which ultimately leads to enhanced cytotoxic function and potentiation of memory T-cells [4-6] (**Figure 1**). On Tregs, GITR has a more complex role where short-term stimulation promotes loss of forkhead box P3 (FoxP3) on Tregs leading to decreased activity of these cells [4, 7], but over stimulation leads to expansion and enhancement of their suppressive activity [8, 9] (**Figure 1**). Thus, at the right level of stimulation, GITR could lead to enhanced effector T cell activity directly, and via inhibition of the suppressive function of Tregs.

This modulatory role of GITR on the immune system has made it an attractive target for cancer immune therapeutics. In murine models, agonistic monoclonal GITR antibodies demonstrated *in vivo* anti-tumor activity and confirmed an associated enhanced proliferation of $CD4^+$ and $CD8^+$ TILs as well as depletion of Tregs [4, 10, 11]. Moreover, the addition of an anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) monoclonal antibody led to a synergistic anti-tumor effect in these models [10]. Unfortunately, despite strong preclinical rationale and data, multiple clinical trials have demonstrated, at best, a modest clinical benefit of

Table 1. Examples of clinical trials assessing GITR as a therapeutic target

Drug	Mechanism	Combination	Phase	GITR expression	Cancer type	Results	NCT number	Ref
Active trials with reported outcomes								
MEDI1873	Hexamieric GITR agonist: IgG Fc domain, coronin 1A trimerization domain & GITR-L domain	Monotherapy	1	Not required for enrollment	Advanced or metastatic solid tumors	ORR=0/40 (0%) No further development planned due to lack of tumor response	NCT02583165	[41]
INCAGN01876 (Ragifilimab)	GITR agonistic monoclonal antibody	Monotherapy	1/2a	Not required for enrollment	Advanced or metastatic solid tumors	ORR=4/100 (4%); All responses at 0.3 mg/Kg dose (4/16 (25%))	NCT02697591	[12]
INCAGN01876	GITR agonistic monoclonal antibody	Plus nivolumab, ipilimumab or both	1/2	Not required for enrollment	Advanced or metastatic solid tumors	Phase 1 ORR ranged from 20-25% when combined with nivolumab and 12.5-25% when combined with ipilimumab (1/4 or 1/5 patients in each cohort) Phase 2 only had response in combination with nivolumab (no ipilimumab combinations were tested): ORR=11/46 (23.9%) in head and neck squamous cell ORR=3/18 (16.7%) in cervical	NCT03126110	[38]
TRX518	GITR agonistic monoclonal antibody	Monotherapy	1	Not required for enrollment	Stage III or IV melanoma + other solid tumors	ORR=1/31 (3.2%)	NCT01239134	[42]
TRX518	GITR agonistic monoclonal antibody	Plus either gemcitabine, pembrolizumab or nivolumab	1	Not required for enrollment	Advanced or metastatic solid tumors	Gemcitabine arm: ORR=1/26 (3.8%) Pembrolizumab arm: ORR=1/25 (4.0%) Nivolumab arm: ORR=1/8 (12.5%)	NCT02628574	[42]
BMS-986156	GITR agonistic monoclonal antibody	± Nivolumab	1/2	Not required for enrollment	Advanced or metastatic solid tumors	Combination Therapy: ORR=21/252 (8.3%) Most responses seen with 240 mg of BMS-986156 plus 240 mg Nivo (ORR=18/200 (9%)) but highest ORR seen with 100 mg of BMS-986156 plus 240 mg Nivo (ORR=1/9 (11.1%)) Monotherapy: ORR=0/34 (0%)	NCT02598960	[43]
MK-4166	GITR agonistic monoclonal antibody	± Pembrolizumab	1	Not required for enrollment	Advanced or metastatic solid tumors (had melanoma cohort)	Combination therapy: ORR=1/45 (2.2%) in dose escalation/confirmation cohort ORR=8/13 (61.5%) in IO naïve melanoma patients in dose expansion cohort ORR=8/20 (40%) in all melanoma patients in dose expansion cohort Monotherapy: ORR=0/48 (0%)	NCT02132754	[13]
GWN323	GITR agonistic monoclonal antibody	± Spartalizumab	1/1b	Not required for enrollment	Advanced solid tumors and lymphomas	Combination arm: ORR=4/53 (7.5%); 1 patient (endometrial Ca) had CR (1/53 (1.9%)) Monotherapy: ORR=0/39 (0%)	NCT02740270	[44]
Active trials without reported outcomes								
INCAGN01876	GITR agonistic monoclonal antibody	Plus anti-PD-1 & autophagosome vaccine (DVP-001)	1	Not required for enrollment	Recurrent or metastatic HNSCC	N=56 Clinical outcomes not published	NCT04470024	
INCAGN01876	GITR agonistic monoclonal antibody	Plus anti-PD-1 and SRS vs. resection	2	Not required for enrollment	Glioblastoma	N=32 Clinical outcomes not published	NCT04225039	
REGN6569	GITR agonistic monoclonal antibody	Plus Cemiplimab	1	Not required for enrollment	Unresectable or metastatic HNSCC	N=85 Clinical outcomes not published	NCT04465487	
BMS-986156	GITR agonistic monoclonal antibody	Plus ipilimumab and nivolumab ± SBRT	1/2	Not required for enrollment	Metastatic solid malignancies	N=60 Clinical outcomes not published	NCT04021043	

ASP1951	GITR agonistic monoclonal antibody	± Pembrolizumab	1b	Not required for enrollment	Advanced or metastatic solid tumors	N=120 Clinical outcomes not published	NCT03799003	
INCAGN01876	GITR agonistic monoclonal antibody	Monotherapy	2	Not required for enrollment	Recurrent or metastatic HNSCC	N=340 Clinical outcomes not published	NCT03088059	
Terminated Trials								
AMG 228	GITR agonistic monoclonal antibody	Monotherapy	1	Not required for enrollment	Advanced or metastatic solid tumors	ORR=0/27 (0%) No evidence of T-cell activation or anti-tumor activity	NCT02437916	[45]
INCAGN01876	GITR agonistic monoclonal antibody	Plus Epacadostat and Pembrolizumab	1/2	Not required for enrollment	Advanced or metastatic solid tumors	N=10 No patients completed trial, 2 terminated by sponsor, 4 subject withdrawal and 4 death	NCT03277352	[46]
MK-1248	GITR agonistic monoclonal antibody	± Pembrolizumab	1	Not required for enrollment	Advanced or metastatic solid tumors	Combination therapy: ORR=3/17 (18%) Monotherapy: ORR=0/20 (0%) Enrollment prematurely discontinued due to "program prioritization"	NCT02553499	[47]
MK-4166	GITR agonistic monoclonal antibody	Plus nivolumab	1	Not required for enrollment	Glioblastoma	N=3 Clinical outcomes not published	NCT03707457	
TRX518	GITR agonistic monoclonal antibody	Plus cyclophosphamide ± Avelumab	1b/2a	Not required for enrollment	Advanced solid tumors (some cohorts ovarian, prostate and breast)	N=10 Clinical outcomes not published	NCT03861403	
OMP-336B11	Fusion protein consisting of two trimeric human GITRLs and a human immunoglobulin Fc domain	Monotherapy	1a	Not required for enrollment	Advanced or metastatic solid tumors	N=24 Clinical outcomes not published	NCT03295942	
Dendritic cell vaccine	Autologous dendritic cells transfected with RNAs encoding melanoma tumor antigens in conjunction with another population encoding GITR-L		1	Not required for enrollment	Metastatic melanoma	N=2 Clinical outcomes not published	NCT01216436	

Abbreviations: GITR, Glucocorticoid-induced tumor necrosis factor related protein; GITR-L, Glucocorticoid-induced tumor necrosis factor related protein ligand; HNSCC, head and neck squamous cell carcinoma; Nivo, nivolumab; ORR, overall response rate; RNA, ribonucleic acid.

GITR agonism. Indeed, overall response rates (ORR) in these studies have ranged between 0-4% when used as monotherapy, and 0-25% in the combination setting (**Table 1**) [12, 13, 28, 31-37]. There were however notable exceptions to these values; a phase 1/2 study of INCAGN01876 (NCT02697591) as monotherapy in advanced solid tumors demonstrated an ORR of 25% (4/16 patients) at the 0.3 mg/Kg dosing, but failed to show any responses at other doses, including much higher doses (up to 10 mg/kg) [12]. Another trial studying MK-4166 (NCT02132754) demonstrated an ORR as high as 61.5% (8/13) in previously untreated melanoma patients; however, this was in combination with nivolumab, and it is well established that melanoma is very sensitive to anti PD-1 treatment. Thus, the contribution of GITR agonism is probably limited in this setting, especially given the ORR of 0% when the GITR agonist was used as monotherapy in the same trial [13].

Multiple reasons could explain the limited responses observed in GITR agonist trials. First, GITR expression was not assessed in any of these trials. Second, these early trials mostly assessed the maximum tolerated dose, but given GITR's complex interaction, this may not be the most efficacious dose as exemplified by the clinical trial studying INCAGN01876, which noted a higher ORR at a lower dose level than the maximum dose tested [12]. These complexities are being addressed and dose selection by minimum anticipated biologic effect or biologically active doses are being explored [14]. Finally, GITR agonism is thought to be limited by T cell exhaustion, notably through the known competitive interaction of CD226 and T-cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) with CD155; while the interaction of CD226 with CD155 leads to effector cell activation, prolonged activation leads to upregulation of TIGIT, which also interacts with CD155 and leads to effector cell inhibition and T cell exhaustion [15]. Interestingly, the addition of an anti-PD-L1 to GITR agonism restores balance between CD226 and TIGIT and leads to a survival benefit in mouse studies [16].

In this study, we sought to explore the differences in GITR ribonucleic acid (RNA) expression patterns between tumor types, as well as correlations with GITR-L and other known immune modulators' RNA expression, in an attempt to explain the variable responses to GITR agonists noted in clinical trials.

Materials and methods

Patients

Overall, 514 patients from the University of California San Diego (UCSD) Moores Cancer Center with various solid tumor histologies were assessed. RNA expression levels from tumor samples were analyzed at OmniSeq (Labcorp) as previously reported [17]. Other relevant variables and patient demographics were collected directly from patient charts. If the same patient had multiple tumor samples evaluated, data from the first sample was used for this analysis. The study was conducted per the guidelines of the UCSD Institutional Review Board (Study of Personalized Cancer Therapy to Determine Response and Toxicity, UCSD_PREDICT, NCT02478931) and investigational interventions for which the all patients consented.

Tissue processing and analysis of relevant markers

Tissue samples were collected and processed (formalin-fixed paraffin-embedded (FFPE)). RNA was extracted and purified from tissue using truXTRAC FFPE extraction kit (Covaris, Inc., Woburn, MA) per manufacturer's instructions. RNA was reconstituted in 50 µL water, and the yield was measured via Quant-iT RNA HS assay (Thermo Fisher Scientific, Waltham, MA), per the manufacturer's instructions. A titer of 10 ng of RNA was deemed acceptable for sequencing and RNA sequencing absolute read was generated with Torrent Suite's plugin immuneResponseRNA (v5.2.0.0). Transcript abundance was normalized to an internal housekeeping gene profile dataset and ranked (0-100 percentile) in a standardized manner; the reference dataset of 735 tumors spanning 35 tumor histologies. In the current investigation, RNA expression of GITR (TNFRSF18; CD357), GITR-L, PD-L1, CTLA4, lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3), OX40, inducible T-cell co-stimulator (ICOS), CD137, TIGIT, CD226, and FoxP3 were assessed. To assess tumor mutational burden (TMB), known to be a predictive factor for immunotherapy response and also for prognosis [18,

19], DNA was extracted employing the truXTRAC FFPE extraction kit (Covaris). For this study, 10 ng of DNA was deemed adequate for preparing DNA libraries, which were created with Ion AmpliSeq targeted sequencing chemistry (Comprehensive Cancer Panel). Samples were enriched/templated utilizing the Ion Chef system and sequenced on the Ion S5XL 540 chip (Thermo Fisher Scientific). TMB is read as mutations/megabase; single nucleotide variants with <5% variant allele fraction, synonymous and germline variants, and indels are eliminated. PD-L1 immunohistochemistry (IHC) was assessed using either the SP142 (Ventana) or 22C3 (Dako) antibodies. Human epidermal growth factor 2 (HER2) status was assessed by IHC and FISH per established guidelines [20], and estrogen receptor (ER) and progesterone receptor (PR) was assessed by IHC per local institutional protocol.

Statistical analysis

RNA expression levels were stratified by high, moderate and low expression. High expression was defined as 75th-100th percentile, moderate expression, defined as 25th-74th percentile, and low expression, defined as 0-24th percentile. The dataset has been previously described [21-23]. Univariate and multivariate odds ratio and *p*-values were calculated using logistic regression with R version 4.2.1 (R statistical computing, Vienna, Austria). GraphPad Prism version 9.5.1 for Windows (GraphPad Software, Boston, Massachusetts USA) was also used for figure creation.

Results

Patients

Overall, 514 patients with various tumor types were evaluated. Median age was 61 years, and ages ranged from 24-93 years. A total of 310 (60.3%) patients were female and 204 (39.7%) were male. Most common tumor types were colorectal (n=140 (27.2%)), pancreatic (n=55 (10.7%)), breast (n=49 (9.5%)), ovarian (n=43 (8.37%)), gastric (n=25 (4.86%)), sarcoma (n=24 (64.7%)), uterine (n=24 (6.47%)) and lung (n=20 (3.89%)). The aforementioned tumor types all had more than 20 patients per tumor type; the remaining 134 patients (26.1%) had malignancies which constituted less than 20 samples per tumor type.

Landscape of GITR and GITR-L RNA expression

RNA expression levels were stratified by high expression (75th-100th percentile), moderate expression (25th-74th percentile), and low expression (0-24th percentile). Overall, 99/514 (19.3%) patients had high GITR expression. Malignancies that had 20 or more representative samples were evaluated for expression and reported. Breast and lung had the largest proportion of patients with high GITR expression, with 38.8% (19/49) and 35% (7/20) of patients respectively. Of all breast cancer patients with high GITR expression who had ER, PR and HER2 data available (n=18), 0% had HER2 expression as determined by IHC and FISH testing (0/18); 38.9% (7/18) were triple negative and 61.1% (11/18) were hormone receptor (HR) positive, HER2 negative. Of the remaining breast cancer patients that did not have high GITR expression, and had ER, PR and HER2 data available (n=28), 50.0% (14/28) were HR positive and HER2 negative, 14.3% (4/28) were HR positive and HER2 positive, 28.6% (8/28) were triple negative and 7.1% (2/28) were HR negative and HER2 positive. All other malignancies evaluated had ≤20% of patients with high GITR expression; 20% (5/25) in gastric, 16.5% (7/43) in ovarian, 14.5% (8/55) in pancreatic, 13.6% (19/140) in colorectal, 12.5% (3/24) in uterine and 4.2% (1/24) in sarcoma (**Figure 2**).

GITR-L expression was also assessed in malignancies that had 20 or more representative samples; highest expression was seen in pancreatic with 38.2% (21/55) of patients expressing high GITR-L. Uterine cancer had the lowest proportion of patients expressing high GITR-L at 12.5% (3/24). All other malignancies had similar proportion of patients with high GITR-L expression; 24% (6/25) in gastric, 20.8% (5/24) in sarcoma, 20.4% (10/59) in breast, 20% (28/140) in colorectal, 20% (4/20) in lung, and 18.6% (8/43) in ovarian had high GITR-L expression (**Figure 2**).

The association between GITR and GITR-L RNA expression were assessed in malignancies that had 20 or more representative samples. Tumors with highest proportion of patients having high GITR expression and low-moderate GITR-L expression were lung and breast, with 30% (6/20) and 30.6% (15/49) respectively,

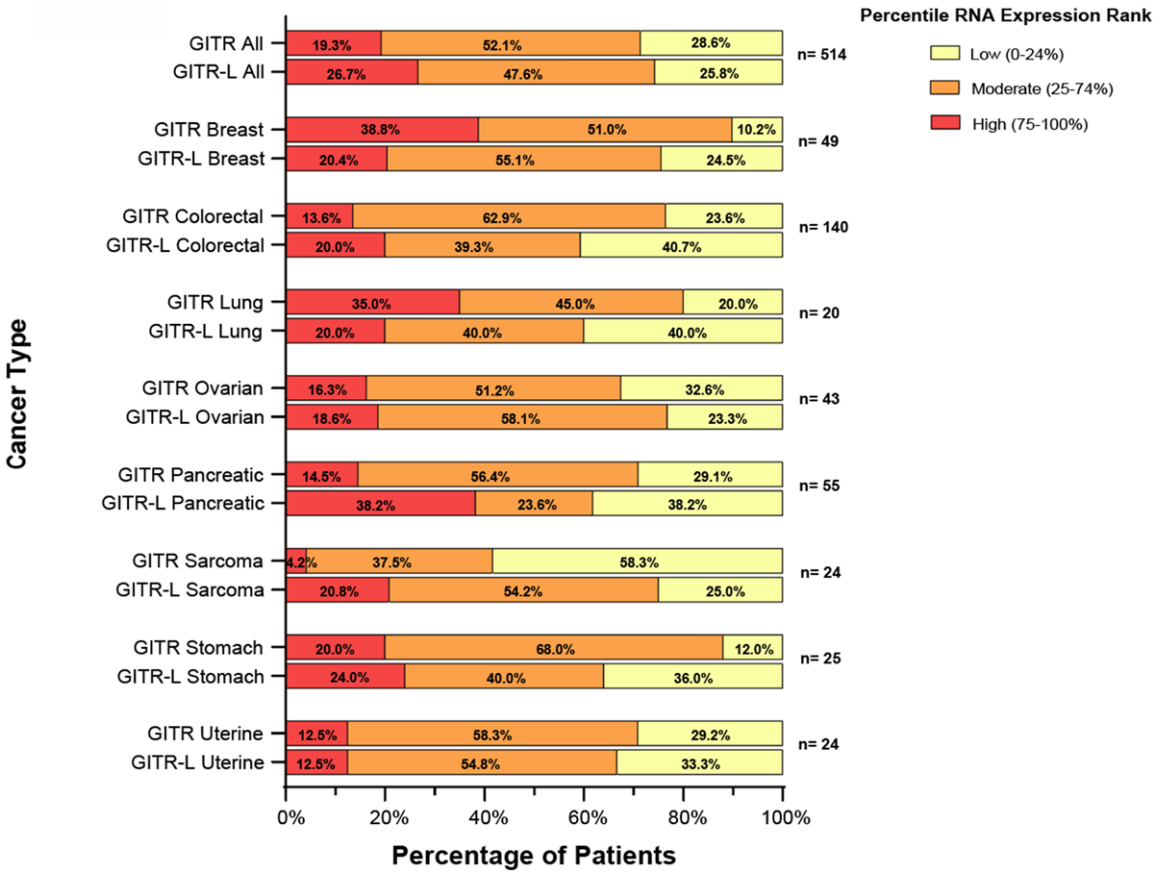

Figure 2. Proportion (%) of patients with GITR and GITR-L RNA expression. Malignancies with >20 representative samples were included. Levels of GITR and GITR-L expression were stratified by high (75th-100th percentile), moderate (25th-74th percentile) and low (0-24th percentile) expression.

representing the only two malignancies tested with >20% of patients having this expression pattern (**Figure 3A**). Of all breast cancer patients with high GITR plus low-moderate GITR-L expression who had ER, PR and HER2 data available (n=14), 0% had HER2 expression as determined by IHC and FISH testing (0/14); 35.7% (5/14) were triple negative and 64.3% (9/14) were hormone receptor positive, HER2 negative. The entire cohort of patients with breast cancer showed HER2 positivity in only 6 of 49 tumors, limiting any implications of this observation. Otherwise, the proportion of patients having high GITR along with low-moderate GITR-L expression were 20% (5/25) in gastric, 14% (6/43) in ovarian, 11.4% (16/140) in colorectal, 7.3% (4/55) in pancreatic, 4.2% (1/24) in sarcoma, and 4.2% (1/24) in uterine (**Figure 3B**).

High GITR RNA expression is associated with breast cancer as well as other clinically relevant immune checkpoint markers

Breast cancer was associated with high GITR expression on both univariate (P<0.001, odds ratio (OR) 3.05, confidence interval (CI) 1.61-5.65) and multivariate analysis (P<0.001, OR 3.79, CI 1.74-8.17). Other malignancies with more than 20 representative samples did not show a statistically significant association with high GITR expression, though lung cancer trended towards significance on univariate analysis (P=0.08, OR 2.35, CI 0.86-5.91) (**Table 2**). Colorectal cancer was found to be negatively associated with high GITR expression on univariate analysis (P=0.47, OR 0.58, CI 0.33-0.97), but was did not show a significant association with high GITR expression on multivariate analysis.

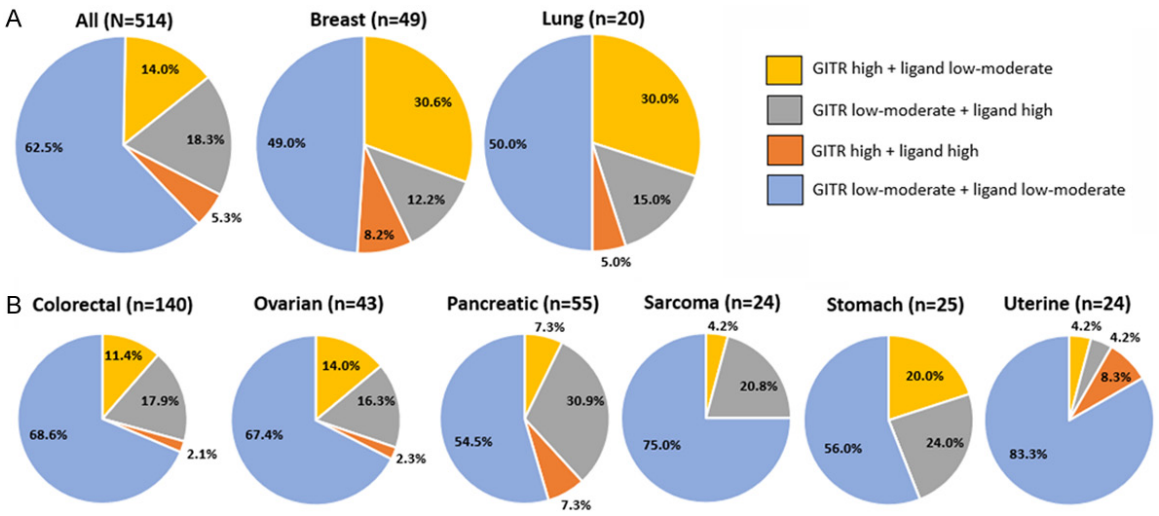

Figure 3. Proportion (%) of patients with different combinations of GITR and GITR-L RNA expression stratified by high and low-moderate expression. GITR RNA expression of 75th-100th percentile was considered high and GITR-L expression of 0-74th percentile was considered low-moderate. A. All malignancies, and malignancies with ≥30% of patients having high GITR RNA expression with low-moderate GITR-L RNA expression. B. All malignancies with >20 samples with <30% of patients having high GITR RNA expression with low-moderate GITR-L RNA expression.

On univariate analysis, high GITR RNA expression showed a statistically significant association with PD-L1 IHC positivity of at least 1% (P<0.001, OR 2.53, CI 1.61-3.97), and high RNA expression of PD-L1 (P<0.001, OR 5.19, CI 3.00-8.96), PD-1 (P<0.001, OR 4.63, CI 2.82-7.60), CTLA4 (P<0.001, OR 6.47, CI 3.91-10.8), LAG-3 (P<0.001, OR 4.04, CI 2.52-6.47), OX40 (P<0.001, OR 4.59, CI 2.88-7.35), ICOS (P< 0.001, OR 4.74, CI 2.76-8.12), CD137 (P< 0.001, OR 3.93, CI 2.32-6.62), TIGIT (P<0.001, OR 5.24, CI 3.22-8.55) and FoxP3 (P<0.001, OR 5.06, CI 3.17-8.12). Low expression (<25th percentile RNA expression rank) of CD226 was negatively associated with GITR high expression (P=0.005, OR 0.48, CI 0.29-0.79) on univariate analysis. On multivariate analysis, only PD-L1 IHC≥1 (P=0.008, OR 2.15, CI 1.22-3.78), high RNA expression of CTLA4 (P=0.05, OR 2.17, CI 1.00-4.71) and OX40 (P=0.001, OR 2.64, CI 1.46-4.73) were significantly associated with high GITR expression (**Table 2**).

Discussion

As a target for cancer therapeutics, GITR has yet to prove itself as multiple clinical trials have shown, at best, a modest response to GITR agonism (**Table 1**). While many variables could be responsible for the limited efficacy noted in GITR agonist trials, a possible contributor is failure to identify the population of patients whose tumors are most likely to respond. There is strong precedence for the ability of biomarkers to predict outcomes with certain treatments. In the immunotherapy field, this is exemplified with universally good responses seen with anti-PD-1/PD-L1 therapy in patients with high TMB [18, 19, 24-27], and/or microsatellite instability (MSI)/deficient mismatch repair (dMMR) [28, 29]. The gene-targeted therapy approach has also shown that selection of patients whose tumors have oncogenic drivers may be critical for efficacy when driver alterations such as *NTRK* fusions [30, 31], *RET* fusions [32, 33], and *BRAF* mutations [34, 35] are targeted, leading to genomic-based tumor-agnostic approvals by the Food and Drug Administration. Moreover, our experience with the I-PREDICT trial has demonstrated that using a personalized N-of-1 combination therapy approach guided by patient genomics leads to improved outcomes [36]. In the current study, we utilized transcriptomic data, which have previously been shown to have utility in treatment selection [37], to explore certain clinical populations and relevant clinical variables that could be informative for GITR agonism in clinical trials.

As part of the current study, exploring trends in GITR RNA expression revealed that breast and

Table 2. Univariate and multivariate logistic regression analysis exploring the relationship of high GITR (>75% percentile rank) RNA expression with relevant clinical and molecular variables (N=514 patients)

Variable		Proportion of High-GITR Expression	Univariate Odds Ratio (95% CI)	Univariate *P*-value	Multivariate Odds Ratio (95% CI)	Multivariate *P*-value	Comments
Age	≥61	21% [55/256]	1.33 (0.86, 2.07)	0.20			
	<61#	17% [44/258]					
Gender	Male	16% [32/204]	0.67 (0.42, 1.07)	0.10			
	Female#	22% [67/310]					
Breast cancer*	Yes	39% [19/49]	3.05 (1.61, 5.65)	<0.001	3.79 (1.74, 8.17)	<0.001	High GITR RNA expression correlates with breast cancer vs. other cancers
	No#	17% [80/465]					
Colorectal cancer	Yes	14% [19/140]	0.58 (0.33, 0.97)	0.047	0.76 (0.39, 1.43)	0.41	-
	No#	21% [80/374]					
Lung cancer	Yes	35% [7/20]	2.35 (0.86, 5.91)	0.08			
	No#	19% [92/494]					
Ovarian cancer	Yes	16% [7/43]	0.80 (0.32, 1.75)	0.61			
	No#	20% [92/471]					
Pancreatic cancer	Yes	15% [8/55]	0.69 (0.29, 1.43)	0.35			
	No#	20% [91/459]					
Sarcoma	Yes	4% [1/24]	0.17 (0.01, 0.84)	0.09			
	No#	20% [98/490]					
Stomach cancer	Yes	20% [5/25]	1.05 (0.34, 2.67)	0.92			
	No#	19% [94/489]					
Uterine cancer	Yes	13% [3/24]	0.59 (0.14, 1.75)	0.40			
	No#	20% [96/490]					
PD-L1 IHC**	≥1	30% [47/156]	2.53 (1.61, 3.97)	<0.001	2.15 (1.22, 3.78)	0.008	High GITR RNA expression correlates with PD-L1 IHC≥1 vs. negative PD-L1 IHC
	<1#	15% [52/357]					
MSI_H***	High	7% [1/15]	0.29 (0.02, 1.49)	0.24			
	Not high#	20% [91/465]					
TMB (muts/MB)****	≥10	15% [5/33]	0.79 (0.26, 1.95)	0.64			
	<10#	18% [77/417]					

Transcriptomics							
PD-L1	≥75	48% [32/67]	5.19 (3.00, 8.96)	<0.001	1.61 (0.74, 3.43)	0.22	-
	<75#	15% [67/447]					
PD-1	≥75	43% [40/93]	4.63 (2.82, 7.60)	<0.001	1.20 (0.53, 2.62)	0.65	-
	<75#	14% [59/421]					
CTLA4	≥75	49% [43/87]	6.47 (3.91, 10.8)	<0.001	2.17 (1.00, 4.71)	0.05	High GITR RNA expression correlates with high CTLA4 RNA expression vs. low-moderate CTLA4 RNA expression
	<75#	13% [56/427]					
LAG3	≥75	39% [45/116]	4.04 (2.52, 6.47)	<0.001	1.60 (0.83, 3.02)	0.15	-
	<75#	14% [54/398]					
TIM3	≥75	27% [24/90]	1.69 (0.98, 2.85)	0.052			
	<75#	18% [75/424]					
OX40	≥75	40% [49/122]	4.59 (2.88, 7.35)	<0.001	2.64 (1.46, 4.73)	0.001	High GITR RNA expression correlates with high OX40 RNA expression vs. low-moderate OX40 RNA expression
	<75#	13% [50/392]					
ICOS	≥75	46% [32/70]	4.74 (2.76, 8.12)	<0.001	0.89 (0.37, 2.09)	0.79	-
	<75#	15% [67/444]					
CD137	≥75	42% [32/77]	3.93 (2.32, 6.62)	<0.001	1.10 (0.48, 2.39)	0.81	-
	<75#	15% [67/437]					
TIGIT	≥75	44% [44/99]	5.24 (3.22, 8.55)	<0.001	1.18 (0.48, 2.84)	0.71	-
	<75#	13% [55/415]					
CD226	<25	13% [23/183]	0.48 (0.29, 0.79)	0.005	1.07 (0.58, 1.94)	0.84	-
	≥25#	23% [76/331]					
FOXP3	≥75	41% [51/123]	5.06 (3.17, 8.12)	<0.001	1.58 (0.80, 3.08)	0.18	-
	<75#	12% [48/391]					
TNFSF18	<25	18% [31/177]	0.84 (0.52, 1.33)	0.47			
	≥25#	20% [68/337]					

Malignancies with >20 samples and relevant molecular variables (on all 514 samples) were analyzed. Multivariate analysis was done on variables that had a *p*-value <0.05 in univariate analysis. #Reference value for logistic regression odds ratio; *18/19 breast cancer patients with high GITR had ER, PR and HER2 status data available. 61.1% (11/18) were HR+/HER2- and 38.9% (7/18) were HR-/HER2-; **One sample was missing PD-L1 IHC data and was omitted from analysis; ***34 samples were missing MSI data and were omitted from analysis; ****64 samples were missing TMB data and were omitted from analysis. Abbreviations: CI, confidence interval; IHC, immunohistochemistry; MSI, microsatellite instability; TMB, tumor mutational burden; HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

lung cancers have a disproportionally higher expression of GITR relative to other malignancies (**Figure 2**). Moreover, in these two malignancies, about 30% of patients had high GITR expression with concomitant low-moderate GITR-L expression (**Figure 3**); theoretically, this scenario - high expression of the receptor but low amounts of ligand - might be most amenable to GITR agonism. Interestingly, none of the patients with breast cancer having high GITR expression had HER2 positivity and 64.3% (9/14) were hormone receptor positive, HER2 negative; this observation that warrants further investigation as immunotherapy has yet to find a role in HR+ and HER2- disease. Moreover, it is worth mentioning that review of the clinical trials in **Table 1** revealed that patients with breast cancer were generally underrepresented (<10% of patients in most trials that reported malignancy types).

High GITR expression has a statistically significant association on univariate analysis with high RNA expression of multiple known immunomodulatory markers, namely, PD-L1, CTLA4, LAG-3, OX40, ICOS, CD137 (4-1BB), TIGIT, as well PD-L1 IHC≥1. This is likely a representation of a "hot" immune repertoire as multivariate analysis revealed a statistically significant association of high GITR expression with only high CTLA4 and OX40 RNA expression and PD-L1 IHC≥1. The correlation of high co-expression of these markers could imply that targeting these three markers in combination may yield better outcomes; indeed, many of the trials assessing GITR agonists have added an anti PD-L1/PD-1 with better responses (**Table 1**) and at least one trial [38] noted higher response rates with addition of an anti-CTLA4 to GITR agonism. Using OX40 agonists in conjunction with GITR agonists has also shown to have a synergistic effect in murine studies [39], though to our knowledge, no clinical trials have been undertaken to assess this combination.

While our study sheds light on potential immunomic expression considerations that may aid in finding a niche for targeting GITR in cancer therapeutics, it has several important limitations. Firstly, patients who were analyzed as part of the database did not have a pre-determined inclusion criterion, and sampling bias could have skewed these results. Secondly, a few malignancies in our database had <20 patients, and some malignancies were not represented at all; thus, our results speak best to the malignancies with more than 20 samples in the queried database. Thirdly, while a minimum number of 20 patients per tumor type was required to proceed with analysis, this is still a fairly low number of samples which may have lacked power to obtain a statistical signal in populations that may be truly associated with higher GITR expression. Fourthly, this analysis explored only mRNA expression levels and does not assess protein levels, nor were we able to segregate expression by cell type. Lastly, though we hypothesized that the presence of high GITR with concomitant low-moderate GITR-L RNA expression may be more amenable to GITR agonism based on the known phenomenon that GITR overstimulation leads to immunosuppression (**Figure 1**), there is no clinical/preclinical data as of yet that implicates this profile in preferential clinical benefit. It is also important to note that the GITR low-moderate plus GITR-L low-moderate expression pattern made up the majority of patients across cancer types (**Figure 3**), a group that warrants further molecular characterization, especially if lack of meaningful clinical benefit with GITR agonism is observed in further studies. Ultimately, while we identified associations between high GITR expression and other targetable immune markers, as well as high GITR expression and certain patient populations, confirming therapeutic benefit with GITR agonism in patients with high GITR expression (and potentially concomitant GITR-L low-moderate expression) requires further prospective trials.

In summary, multiple reasons likely account for the suboptimal outcomes from current trials exploring therapeutics targeting GITR expression in solid tumors. While some of these reasons may be related to the complexities of drug design [40], dosing, and timing of administration relative to other immune agents, our current study sheds light on other intrinsic variables such as the heterogeneity of expression of GITR and GITR ligand between and within cancer types. These variable expression patterns merit further study as to whether they may influence responsiveness to GITR agonism. The significant association of high GITR expression with breast cancer, as well as the observation that almost a third of breast can-

cer patients in our cohort had high GITR expression with concomitant low-moderate GITR-L expression, and similar finding in lung cancer, may also be of interest for clinical trial targeting. The results herein also support a combination approach to GITR agonism, specifically with agonists of OX40 and antagonists of PD-L1 and CTLA4, given that PD-L1 IHC≥1 and high RNA expression of OX40 and CTLA4 were independently associated with high GITR RNA expression.

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