

# Molecular and Clinical Characterization of LAG3 in Breast Cancer Through 2994 Samples

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## Research

**Keywords:** Cancer immunotherapy, CD223, LAG3, Immune response, Inflammatory activity

**Posted Date:** June 19th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-36422/v1>

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# Abstract

**Background** Despite the promising impact of cancer immunotherapy targeting CTLA4 and PD1/PDL1, a large number of cancer patients fail to respond. LAG3 (Lymphocyte Activating 3), also named CD233, is a protein coding gene served as alternative inhibitory receptors to be targeted in the clinic. The impact of LAG3 on immune cell populations and co-regulation of immune response in breast cancer remained largely unknown.

**Methods** To characterize the role of LAG3 in breast cancer, we investigated transcriptome data and associated clinical information derived from a total of 2994 breast cancer patients.

**Results** We observed that LAG3 was closely correlated with major molecular and clinical characteristics, and was more likely to be enriched in higher malignant subtype, suggesting LAG3 was a potential biomarker of triple-negative breast cancer. Furthermore, we estimated the landscape of relationship between LAG3 and ten types of cell populations in breast cancer. Gene ontology analysis revealed LAG3 were strongly correlated with immune response and inflammatory activities. We investigated the correlation pattern between LAG3 and immune modulators in pan-cancer, especially the synergistic role of LAG3 with other immune checkpoints members in breast cancer.

**Conclusions** LAG3 expression was closely related to malignancy of breast cancer and might serve as a potential biomarker; LAG3 might plays an important role in regulating tumor immune microenvironment, not only T cells, but also other immune cells. More importantly, LAG3 might synergize with CTLA4, PD1/PDL1 and other immune checkpoints, thereby lending more evidences to combination cancer immunotherapy by targeting LAG3, PD1/PDL1, and CTLA4 together.

## Research In Context

LAG3, also named CD233, is a protein coding gene served as an alternative inhibitory receptor to be targeted in the clinic. A plethora studies have been focused on the impact of LAG3 on T cell immunity. However, the impact of LAG3 on other immune cell populations and immune modulators, and its expression pattern has so far remained unclear in breast cancer tissue. To clarify the relationship between LAG3 expression and clinical practice, we comprehensively analyzed the molecular and clinical characteristics of LAG3 expression in large sample-sized breast cancer. High level of LAG3 expression was associated with malignant entities. Gene ontology analysis indicated that LAG3 played a pivotal role in the regulation of immune responses and inflammatory activities. LAG3 might be not only involved in T cell immunity, but also other immune cells induced immunity. Moreover, our results also suggested the synergistic role of LAG3 with other immune checkpoints members, thereby lending support to combination cancer immunotherapy by targeting LAG3 and other immune checkpoint molecules.

## 1. Introduction

Breast cancer is the most common malignancy and also the leading cause of death in women worldwide (1). Despite a great deal of progresses in comprehensive therapy, such as breast conservative surgery/radical mastectomy, neoadjuvant/adjuvant chemotherapy, adjuvant radiotherapy, targeted therapy, endocrine therapy and emerging immunotherapy, approximately 46 million women die from breast cancer each year and patients who suffer from recurrence and metastasis of breast cancer still have a relatively short median survival time due to the aggressiveness of tumors, the low response rate to immunotherapy, and the resistance medical treatments (2).

In the past decade, many studies had focused on the immunotherapy of various cancer, and benefits from blockage of the interaction between programmed death-1 (PD-1) and ligand-1 (PD-L1) to inhibit the suppression of T cell immune responses have been observed (3). Meanwhile, several clinical trials of PD-1/PD-L1 targeting in breast cancer have also been initiated (4). However, given that the objective response rate has been 13%~56% and the complete response rate has been 1%~16%, there was limited success of such emerging therapy, especially for breast cancer (5–11). Therefore, the acknowledgment of the immunotherapy is not fully understood and more research is needed.

Many previous studies have discovered several negative co-stimulatory molecules like the programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) axis, lymphocyte activation gene-3 (LAG3), cytotoxic T lymphocyte associated antigen-4 (CTLA4), T-cell immunoglobulin and mucin domain protein 3 (TIM3) and so on, which participate in inhibiting T cells and facilitating different tumor cells to escape singly or jointly (3, 12–14). Over-expression of inhibitory receptors (IRs) is significant to balance co-stimulatory, receptor activity and limit T-cell activation, thus helps to prevent autoimmunity, autoinflammation, and tissue damage. Despite the impressive impact of CTLA4 and PD1-PDL1-targeted cancer immunotherapy, serving as a cancer immunotherapy target, LAG3 (also named CD223) is the third IR to be targeted in the clinic due to its negative regulatory role on T cells and its capacity, combined with PD1, to mediate a state of exhaustion (15), consequently attracting considerable interest and scrutiny (12). As a protein coding gene, LAG3 belongs to Ig superfamily and contains 4 extracellular Ig-like domains. LAG3 is highly expressed on activated human T and NK cells as well as tumor infiltrating lymphocytes (TILs) in various tumors. Previous studies published that serving as an inhibitory receptor on antigen activated T-cells, LAG3 delivered T cell inhibitory signals upon binding to ligands, such as FGL1 (by similarity) (16–18). LAG3 was also suggested to be spatially associated with the T-cell receptor (TCR), especially with CD3-TCR in the immunological synapse and directly inhibited T-cell activation (by similarity) (12). In addition, LAG3 negatively regulated the activation, proliferation, homeostasis and effector function of both CD4(+) and CD8(+) T-cells, and immune tolerance was also mediated by LAG3: constitutively expressed on a subset of regulatory T-cells (Tregs) and consequently contributed to their suppressive function (by similarity) (16–18). Apart from that, it is also involved in inhibiting antigen-specific T-cell activation in synergy with PDCD1/PD-1, which was possibly by acting as a co-receptor for PDCD1/PD-1 (by similarity), and in influencing the therapeutic effect of blocking one of them (12). Not only T cells. LAG3 also acted as a negative regulator of plasmacytoid dendritic cell (pDCs) activation (by similarity), and it also had the potential to bind MHC class II (MHC-II), the precise role of MHC-II-binding is however remained unclear (12).

Previous studies published that LAG3 suppresses T cell activation and the anti-tumor responses in vitro in xx tumor (19–21). However, they did not show the specific expression pattern of LAG3, and its potential impact on other immune cell populations and immune modulators. In the present study, we systematically investigated LAG3 related transcriptome profile, and revealed its potential role in inducing immune response and inflammatory activities, as well as its potential relationship with immune modulators. This study is the first integrative study characterizing landscape of LAG3 expression in breast cancer both molecularly and clinically.

## **2. Materials And Methods**

### **2.1. Data Collection**

TCGA dataset was downloaded through GDCRNATools (access date: Feb 01, 2020) (22). Raw counts data was normalized through TMM implemented in edgeR (23) and was then transformed by voom in limma (24), and only genes with cpm > 1 in more than half of the samples were kept. Sieved TCGA breast cancer clinical data was kindly provided by Dr. Hai Hu and Dr. Jianfang Liu in Chan Soon-Shiong Institute of Molecular Medicine at Windber. HER2 status was recalled using DNA copy number for cases without an IHC or FISH status. Standardized survival data was retrieved from TCGA Pan-Cancer Clinical Data Resource (TCGA-CDR) (25). METABRIC dataset (26) containing a total of 1904 cases were retrieved from cBioPortal database (access date: Feb 01, 2019).

### **2.2. Bioinformatics analysis**

The biological functions of the genes correlated with LAG3 analyzed using clusterProfiler package (27), GO terms and KEGG pathways with adjusted P-value less than 0.05 were considered to be significant. Immunologically related genes were collected from The Immunology Database and Analysis Portal (ImmPort) database (28). the absolute abundance of eight immune and two stromal cell populations using Microenvironment Cell Populations-counter method (29). Gene set variation analysis (GSVA) analysis (30) was performed to estimate the abundance of GO gene sets related with special immune functions and inflammatory metagenes (31). Correlation between LAG3 and immune modulators in pan-cancer were analyzed using TISIDB database (32), an integrated repository portal for tumor-immune system interactions. Spearman correlation analyses were performed to evaluate the correlations between LAG3 and metagenes and special immune functions.

### **2.3. Statistical analysis**

Correlations between continuous variables were assessed by Spearman correlation analyses. Differences in variables between groups were evaluated through the Student t-test, one-way ANOVA, or Pearson's Chi-squared test. All statistical tests were performed using R (version 3.6.0; <https://www.r-project.org/>). Other statistical calculations and graphical work were performed using several packages including ggplot2(33), pheatmap, pROC(34), circlize (35), and corrgram(36). P-value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

## 3. Results

### 3.1. Associations of LAG3 expression with clinical and molecular characteristics in breast cancer

To characterize the association between LAG3 expression and clinical characteristics of breast cancer patients. We dichotomised patients into low- and high-expression groups according to median cut of LAG3 expression. Associations of LAG3 expression and clinical characteristics in both TCGA cohort (n = 1090) and METABRIC cohort (n = 1904) were listed in Tables 1 and 2. LAG3 was associated with AJCC stage, ER, PR, HER2 status in both TCGA and METABRIC dataset, and was associated with T stage in TCGA, as well as age, tumor size and tumor grade in METABRIC. Subsequently, we further explored the expression pattern of LAG3 across various molecular and clinical characteristics. We found LAG3 was up-regulated in breast cancer tissues when compared with normal tissues in TCGA dataset (Fig. 1K). We observed LAG3 expression was up-regulated in ER- group and PR- group in both TCGA and METABRIC databases (Fig. 1A-D). LAG3 was up-regulated in HER2- group in METABRIC database, but this phenomenon was however not observed in TCGA dataset (Fig. 1E and F). LAG3 was over-expressed in higher tumor stage compared with stage I, but was not significant in stage IV (Fig. 1G and H). We found LAG3 was enriched in basal, HER2-enriched and luminal A (LumA) subtype, but not luminal B (LumB) subtype, and these results can be mutually validated in TCGA and METABRIC. Furthermore, we found higher expression of LAG3 was detected in higher tumor grade (Fig. 1L). These results were further validated in an independent microarray datasets derived from GBO database (n = 1881) (37), and correlation analysis revealed that LAG3 expression was strongly correlated with immune response gene modules, which suggest might play important roles in immune related functions. In summary, these findings indicated that the high expression of LAG3 predicted a high malignant breast cancer.

Table 1  
Association between LAG3 mRNA expression and clinicopathologic characteristics in TCGA cohort.

	Expression			P-value
	Total (n = 1090)	LAG3 high (n = 545)	LAG3 low (n = 545)	
<b>Age (years)</b>				
>=55	517 (47.4%)	258 (47.3%)	259 (47.5%)	0.952
< 55	573 (52.6%)	287 (52.7%)	286 (52.5%)	
<b>T stage</b>				
T1	279 (25.6%)	118 (21.7%)	161 (29.5%)	0.016
T2	631 (57.9%)	339 (62.2%)	292 (53.6%)	
T3	137 (12.6%)	71 (13.0%)	66 (12.1%)	
T4	40 (3.7%)	16 (2.9%)	24 (4.4%)	
Missing	3 (0.3%)	1 (0.2%)	2 (0.4%)	
<b>N stage</b>				
N0	514 (47.2%)	256 (47.0%)	258 (47.3%)	0.114
N1	360 (33.0%)	172 (31.6%)	188 (34.5%)	
N2	120 (11.0%)	67 (12.3%)	53 (9.7%)	
N3	76 (7.0%)	44 (8.1%)	32 (5.9%)	
Missing	20 (1.8%)	6 (1.1%)	14 (2.6%)	
<b>M stage</b>				
M0	907 (83.2%)	447 (82.0%)	460 (84.4%)	0.414
M1	22 (2.0%)	10 (1.8%)	12 (2.2%)	
Unknown	161 (14.8%)	88 (16.1%)	73 (13.4%)	
<b>AJCC stage</b>				
I	181 (16.6%)	72 (13.2%)	109 (20.0%)	0.027
II	621 (57.0%)	324 (59.4%)	297 (54.5%)	
III	250 (22.9%)	133 (24.4%)	117 (21.5%)	
IV	20 (1.8%)	9 (1.7%)	11 (2.0%)	

	Expression			
Missing	18 (1.7%)	7 (1.3%)	11 (2.0%)	
<b>ER status</b>				
Negative	236 (21.7%)	169 (31.0%)	67 (12.3%)	< 0.001
Positive	803 (73.7%)	355 (65.1%)	448 (82.2%)	
Unknown	51 (4.7%)	21 (3.9%)	30 (5.5%)	
<b>PR status</b>				
Negative	343 (31.5%)	221 (40.6%)	122 (22.4%)	< 0.001
Positive	694 (63.7%)	302 (55.4%)	392 (71.9%)	
Unknown	53 (4.9%)	22 (4.0%)	31 (5.7%)	
<b>HER2 status</b>				
Negative	895 (82.1%)	438 (80.4%)	457 (83.9%)	0.006
Positive	168 (15.4%)	99 (18.2%)	69 (12.7%)	
Unknown	27 (2.5%)	8 (1.5%)	19 (3.5%)	

Table 2  
Association between LAG3 mRNA expression and clinicopathologic characteristics in METABRIC cohort.

	Expression			P-value
	Total (n = 1904)	LAG3 high (n = 952)	LAG3 low (n = 952)	
<b>Age (years)</b>				
>=55	952 (50.0%)	511 (53.7%)	441 (46.3%)	< 0.001
< 55	952 (50.0%)	441 (46.3%)	511 (53.7%)	
<b>Tumor size</b>				
>=2 cm	592 (31.1%)	268 (28.2%)	324 (34.0%)	0.021
< 2 cm	1292 (67.9%)	673 (70.7%)	619 (65.0%)	
Missing	20 (1.1%)	11 (1.2%)	9 (0.9%)	
<b>AJCC stage</b>				
0	4 (0.2%)	1 (0.1%)	3 (0.3%)	0.003
I	475 (24.9%)	209 (22.0%)	266 (27.9%)	
II	800 (42.0%)	426 (44.7%)	374 (39.3%)	
III	115 (6.0%)	69 (7.2%)	46 (4.8%)	
IV	9 (0.5%)	3 (0.3%)	6 (0.6%)	
Missing	501 (26.3%)	244 (25.6%)	257 (27.0%)	
<b>Tumor Grade</b>				
I	165 (8.7%)	31 (3.3%)	134 (14.1%)	< 0.001
II	740 (38.9%)	288 (30.3%)	452 (47.5%)	
III	927 (48.7%)	598 (62.8%)	329 (34.6%)	
Missing	72 (3.8%)	35 (3.7%)	37 (3.9%)	
<b>ER status</b>				
Negative	445 (23.4%)	334 (35.1%)	111 (11.7%)	< 0.001
Positive	1459 (76.6%)	618 (64.9%)	841 (88.3%)	
<b>PR status</b>				
Negative	895 (47.0%)	568 (59.7%)	327 (34.3%)	< 0.001

		Expression		
Positive	1009 (53.0%)	384 (40.3%)	625 (65.7%)	
<b>HER2 status</b>				
Negative	1668 (87.6%)	786 (82.6%)	882 (92.6%)	< 0.001
Positive	236 (12.4%)	166 (17.4%)	70 (7.4%)	

## 3.2. LAG3 was a potential biomarker for TNBC subtype

To further explore the association of LAG3 expression and breast cancer malignancy, we compared the expression of LAG3 between TNBC and None-TNBC group. We observed LAG3 was significantly up-regulated in TNBC group in both TCGA (n = 1090) and METABRIC (n = 1904) databases (Fig. 3A and B). To further validate this finding, ROC curve analyses for LAG3 expression and TNBC subtype of all breast cancer were performed. Our results indicated area under the curve (AUC) were up to 0.707 and 0.726 in TCGA and METABRIC datasets, respectively (Fig. 3C and D). These findings suggested that LAG3 might play a pivotal role in the progression of breast cancer. Meanwhile, LAG3 might serve as a potential biomarker for TNBC.

## 3.3. LAG3 was closely related to immune functions in breast cancer

To further explore the biological functions of LAG3 in breast cancer, we sieved 746 genes and 582 genes which were strongly correlated with LAG3 by Spearman correlation analysis ( $|R| > 0.4$  and  $P < 0.05$ ) in TCGA and METABRIC datasets, respectively. Subsequently, GO and KEGG functional enrichment analyses were performed to understand the biological roles of LAG3. Consistent with aforementioned results derived from a 1881-sample microarray dataset, GO analyses also revealed that genes correlated with LAG3 were mainly enriched in biological processes related with immune response and inflammatory activities, especially in regulation of T cell, leukocyte, and lymphocyte, and these results can be mutually validated in TCGA and METABRIC datasets (Fig. 4A and B). Meanwhile, KEGG analysis revealed that LAG3 related genes were enriched in pathways related with T cell, PD-L1 expression and PD-1 checkpoint pathway in cancer, Natural killer cell mediated cytotoxicity, Antigen processing and presentation in both TCGA and METABRIC datasets (Fig. 5A and B). These findings further implicate the important role of LAG3 in mediating immune related functions in breast cancer progression.

## 3.4. LAG3 related immune response

To further clarify the role of LAG3 in the immune response in breast cancer, we collected a total of 4723 immunologically related genes from The Immunology Database and Analysis Portal (ImmPort) database. We sieved the genes that were most relevant to LAG3 (Spearman  $|R| > 0.4$ ,  $P < 0.05$ ) to draw the

heatmaps. We found 322 and 254 immunologically related genes were positively correlated with LAG3 in TCGA and METABRIC, respectively, while only 25 and 10 immunologically related genes were negatively correlated with LAG3, respectively (Fig .6A and B). These results revealed that LAG3 is positively correlated with most relevant immune responses while negatively correlated with a small number of immune responses in breast cancer.

### 3.5. Association of LAG3 expression and immune cell populations

To further understand immune regulatory role of LAG3 in breast cancer, we estimated the absolute abundance of eight immune and two stromal cell populations from transcriptome data through Microenvironment Cell Populations-counter method developed by Etienne Becht et al (29). Interestingly, we observed LAG3 expression was positively correlated with T cells, CD8 T cells, Cytotoxic lymphocytes, NK cells, B lineage, Monocytic lineage and Myeloid dendritic cells, but not Neutrophils, Endothelial cells and Fibroblasts (Fig. 7A and B). Especially, LAG had the strongest correlation with T cells, indicating the important role of LAG3 in T cell induced immune functions in breast cancer, and the detailed correlation coefficients between LAG3 and aforementioned cell abundance were listed in Table 3. These results can be mutually verified in TCGA and METABRIC datasets.

Table 3  
Association between LAG3 mRNA expression and immune cell populations in TCGA and METABRIC databases.

Gene		METABRIC		TCGA	
		rho	P-value	rho	P-value
rho	T cells	0.71	< 0.001	0.71	< 0.001
rho1	CD8 T cells	0.39	< 0.001	0.65	< 0.001
rho2	Cytotoxic lymphocytes	0.70	< 0.001	0.63	< 0.001
rho3	NK cells	0.43	< 0.001	0.64	< 0.001
rho4	B lineage	0.52	< 0.001	0.48	< 0.001
rho5	Monocytic lineage	0.58	< 0.001	0.61	< 0.001
rho6	Myeloid dendritic cells	0.28	< 0.001	0.38	< 0.001
rho7	Neutrophils	-0.08	< 0.001	0.12	< 0.001
rho8	Endothelial cells	-0.09	< 0.001	-0.08	0.006
rho9	Fibroblasts	-0.19	< 0.001	-0.07	0.014

### 3.6. The relationship between LAG3 expression and immune modulators in pan-cancer

To further understand the role of LAG3 in regulating immune microenvironment in pan-cancer, we investigated the relationships between LAG3 expression and three types immune modulators described in Charoentong's study (38) through TISIDB database, an integrated repository portal for tumor-immune system interactions. Intriguingly, similar correlation pattern between immune modulators and LAG3 was observed in a total of 30 cancer types, that is, most of immunoinhibitors and immunostimulators were positively correlated with LAG3 (Fig. 8–9), while a minority of immunoinhibitor and immunostimulators were negatively correlated with LAG3. More interestingly, we found LAG3 was positively correlated with almost all MHC molecules in pan-cancer (Fig. 10). These findings suggest that LAG3 might regulating tumor immune microenvironment by synergizing with other immune modulators.

### **3.7. LAG3 is synergistic with other checkpoint members in tumor induced immune response**

To further confirm the synergistic role of LAG3 breast cancer induced immune response, we evaluated the correlations between LAG3 and other checkpoint members (Fig .11A-D). Strong correlations were observed between LAG3 and other checkpoint members. LAG3 was positively correlated with TIGIT ( $r = 0.723$ ,  $r = 0.465$ , TCGA and METABRIC respectively), CD274 (PD-L1) ( $r = 0.592$ ,  $r = 0.365$ ), CD28 ( $r = 0.496$ ,  $r = 0.364$ ), CD40 ( $r = 0.742$ ,  $r = 0.607$ ), CD48 ( $r = 0.636$ ,  $r = 0.652$ ) and other checkpoint molecules including CD27 ( $r = 0.647$ ,  $r = 0.594$ ), CD86 ( $r = 0.614$ ,  $r = 0.609$ ), CTLA4 ( $r = 0.762$ ,  $r = 0.722$ ), ICOS ( $r = 0.754$ ,  $r = 0.744$ ) and IDO1 ( $r = 0.756$ ,  $r = 0.696$ ).

### **3.8. The relationship between LAG3 and special cell immune response**

Previous studies have reported the inhibitory role of T cell activation (39), but it was remained unclear whether LAG3 plays a same role in breast cancer and whether LAG3 have impact on other immune cells. To elucidate the relationship between LAG3 and specific immune response in breast cancer, GSVA analysis was performed. Strong correlations between LAG3 and T cell immunity and B cell immunity were observed (Fig .12A and B). LAG3 was positively correlated with T-helper 1 type immune response, regulation of T cell differentiation, regulation of T cell activation, as well as alpha-beta T cell activation. Meanwhile, LAG3 was positively correlated with B cell mediated immunity, B cell activation and B cell receptor signaling pathway. Importantly, these results can be validated mutually in TCGA and METABRIC databases. These findings suggest LAG3 might also play an inhibitory role in T cell immune tumor in breast cancer, and is also likely to have impact on B cell immunity.

### **3.9. The relationship between LAG3 and inflammatory activities**

To further revealed LAG3-related inflammatory activities, A total of 104 genes derived from seven clusters were defined as metagenes by Gene Sets Variation Analysis (GSVA) (31), detailed gene lists were listed in Table S1. representing different types of inflammation and immune. We found LAG3 were positively

correlated with LCK, HCK, MHC-I, MHC-II, STAT1, and interferon, but not IgG (Fig .12C and D). Among these seven clusters, LAG3 had the strongest correlation with LCK metagenes. More importantly, these results can be mutually verified in TCGA and METABRIC databases. These findings further suggest LAG3 played important immune and inflammatory functions in breast cancer.

## 4. Discussion

As a novel therapeutic approach, immune checkpoint blocking therapy, which can reactivate T cell immune responses to tumor cells and break tumor immune suppression, has achieved marked success in preclinical or clinical trials of many malignant tumors(5, 7, 8, 11, 40), and the most extensively used immune checkpoint inhibitors for the study and application of cancer therapy include PD-1 and inhibitors of its ligand PD-L1, as well as CTLA-4. However, the objective response rate has been 13%~56% and the complete response rate has been only 1%~16%, which presents a frustrating challenge, especially for breast cancer (5–11). Therefore, it is urgent to make further progress on the tumor microenvironment to explore alternative or facilitating therapeutic targets. Many recent studies have shown a special correlation between LAG3 and PD-1 of T cell inhibition in various diseases (12, 41), such as in viral infection (12, 42), chronic tuberculosis infection (43), plasmodium infection (44), chronic lymphocytic leukaemia (45), ovarian cancer (46), and so forth, yet the co-expression and effect of LAG3 and PD-1 on T cells in breast cancer patients are still not very clear. To figure out the molecular and clinical relationship between LAG3 expression and immune activities in breast cancer will greatly promote the establishment and progress of a novel therapeutic target as well as optimize the current therapeutic strategies.

In this study, we systematically analyzed the expression of LAG3 in breast cancer. We found that LAG3 was upregulated in breast cancer tissue, especially enriched in basal, HER-2 enriched and LumA subtype, as well as in patients with higher tumor grade. LAG3 also had potential to serve as valuable biomarker for TNBC subtype. Besides, previous studies also found that the presence of LAG3 + intra-epithelial tumor infiltrating lymphocytes (iTILs) was significantly related to younger age, large tumor size, ER/PR negativity and high Ki67 proliferation index (47, 48). All these results seemed to indicate that the high expression of LAG3 predicted a high malignant breast cancer. However, some studies had come to the seemingly opposite conclusion that high expression of LAG3 is associated with favorable overall survival in several solid tumors including ovarian, gastric, lymphoma, NSCLC, colorectal, and renal cancers (49), as well as in breast cancer. To make a reasonable explanation, we need to focus on the important immunologic role of LAG3.

Under physiological conditions, LAG3 is expressed on membrane of activated human T cells, NK cells, B cells and DCs (50–53), and it is an activation marker for CD4 + and CD8 + T cells. In tumor patients, LAG3 is expressed on surface of TILs (54, 55). Early studies suggested that LAG3 was a negative regulator of T-cell activation, and the regulation of T cells immune response mainly by following three aspects. Firstly, the proliferation and activation of T cells were inhibited directly by negative regulation. Previous studies showed that LAG3 was a negative regulator of T-cell activation, and blockade of LAG3 functioning on human CD4 clones resulted in enhanced cell proliferation with an elevated production of IFN- $\gamma$ , TNF $\alpha$ , IL-

2 and IL-4 (16). Furthermore, there was a largely conserved motif named KIEELE mediated a cell intrinsic signal, which is thought to be essential for the negative regulatory function of LAG3 on T cells (56). A more specific role for LAG3 on CD8 + T cells was demonstrated in a model of self-tolerance. Grosso JF et al. found that adoptively transferred LAG3<sup>-/-</sup> HA-specific CD8 + T cells expanded and produced large amounts of IFN $\gamma$ , indicating that LAG3 limited self-tolerance (52). Moreover, they also found that these CD8 + T cells regained effector function, as was shown with an increased number of IFN $\gamma$ -producing cells. Therefore, hypothesis was that not dependent on CD4 + T cells, the effect induced by blocking LAG3 was shown to be a CD8 + T-cell intrinsic effect (52). Secondly, T cell immune response was suppressed indirectly by promoting inhibitory function of regulatory T cells (Treg). Recent studies have shown that LAG3 promoted the differentiation of Tregs, while its blockade inhibited the induction of Tregs (57). This study also illustrated that CD4 + T cells were skewed into a Th1 phenotype by blockade or genetic deletion of LAG3, with LAG3 limiting IL-2 and STAT5 signaling that modulated the ability to be suppressed by Tregs. Thirdly, T cell activation was prevented by regulating antigen presenting cells (APC) (14), which was also supported by our finding that LAG3 had close correlation with antigen processing and presentation pathways. Published studies also showed that LAG3 may be involved in mediating bidirectional signaling into the interacting APCs. DCs activation was shown to be inhibited by MHC class II binding to LAG3-expressing Tregs, thereby their maturation was suppressed (58). Interestingly, previous studies have been focused on the impact of LAG3 on T cell immunity, whether LAG3 have impact on other immune response and immune cell populations was unclear. Herein, we found that LAG3 was positively correlated with B cell mediated immunity, B cell activation, B cell receptor signaling pathway and natural killer cell mediated cytotoxicity pathways. Consistent to our observations, previous studies indicated that LAG3 expression was also related to NK cells and activated B cells in a T cell-dependent manner (59).

Besides, in terms of immune cells, we observed LAG3 expression had strongest correlation with T cells (especially CD8 + T cells), followed by plasmacytoid dendritic cells, NK cells, monocytic lineage and B lineage. In this section, as the relationship between LAG3 and T cells was already mentioned above, the correlation with other immune cells is focused. LAG3 is constitutively expressed on plasmacytoid dendritic cells (pDCs) at a much greater level than any other cell type (60), while LAG3 is not expressed on any lymphoid DC and myeloid subset. When compared with wildtype pDCs, LAG3- pDCs showed enhanced expansion following CpG stimulation *in vivo*, but had no altered expression profile of activation markers, including differential cytokine production or CD80/86 and MHC class II (60). As was expounded in earlier studies, in humans, LAG3 + pDCs were found to be involved in the melanoma environment and interacted with HLA-DR-expressing tumor cells *in vivo*. Besides, *in vitro* it was also shown that as the result of the stimulation of MHC class II-expressing melanoma cells, LAG3 + pDCs were able to mature and produce IL-6 (61). Therefore, there was a hypothesis that LAG3 + pDCs may indirectly drive myeloid-derived suppressor cells (MDSCs)-mediated immunosuppression through MHC class II + melanoma cells engagement. LAG3 is also found expressed on NK cells (~ 10%) and invariant NKT cells (19). As a result of LAG3 signaling, proliferation of activated NKT cells was reduced, resulting in cell cycle arrest in the phase S (62). Moreover, researchers also found that overexpression of LAG3 was associated with

impaired iNKT cytokine production (IFN $\gamma$ ) during chronic HIV infection, nevertheless this was not discovered on other T-cell subsets (63). As for monocytic lineage, one previous study had suggested that a soluble monomeric form of LAG3 (sLAG3), generated by alternative splicing, impaired the differentiation of monocytes into DCs and macrophages, which subsequently had diminished its immunostimulatory capacity (64). Moreover, another previous study had reported that at the end of IMP321 (a LAG3 antagonist) treatment, there was a 50% objective tumor response and decreased tumor size related with an increase in the absolute number of monocytic cells (65). The role of LAG3 on B cells is controversial since analysis was limited and expression was only reported in a single study (44). In short, LAG3 exerts differential inhibitory impacts on various types of lymphocytes. Except for some relatively deep and detailed researches towards T cells, the functional role and mechanism of LAG3 on other immune cells are not fully understood and well established, and further studies are needed to enrich this field.

Back to the question posed at the beginning of this section, LAG3 expression was found to be associated with poor clinicopathological factors and eliciting immune suppressive function, supporting the hypothesis that the expression of LAG3 in breast cancer patients should lead to poor survival. However, inconsistent with the present results, the findings of several previous studies indicated a favorable association between high expression of LAG3 and cancer-specific survival, especially in ER-, HER2 + and basal-like subtypes (47, 66, 67). Interestingly, another published study found that serum LAG3 had a close correlation with prolonged survival in ER + patients (42). These results implied a complicated relationship between LAG3 expression, clinical characteristics and the prognosis in breast cancer. One possible explanation is that presence of LAG-3 expressing TILs may actually indicate that there is an ongoing cancer-immune interaction (47), a phenotype defined as an inflamed tumor (68), and it usually signified a somewhat improved prognosis. Additionally, another study also illustrated that LAG3 expression by engineered tumor cells efficiently promotes and facilitates activation, intra-tumoral recruitment, and Th1 commitment of APCs, which results in a large intra-tumoral influx of specific and non-specific reactive cells, as well as the release of immunoregulatory and cytotoxic mediators (69). Consequently, further studies are encouraged and needed to focus on this controversial problem.

Despite the promising impact of cancer immunotherapy targeting CTLA4 (like ipilimumab) and PD1/PDL1 (like pembrolizumab), with the in-depth research, the side effects and resistance of these drugs have gradually emerged (70, 71). Moreover, a large number of cancer patients fail to respond: the response rate of ipilimumab was only 15%, and that of PD-1/PD-L1 inhibitors was less than 40% (72). In this study, we found that LAG3 had close correlation with PD-L1 expression and PD-1 checkpoint pathway in cancer, and strong correlations were observed between LAG3 and other checkpoint members, such as CTLA4, TIGIT, CD28, CD40, CD48 and other checkpoint molecules including CD27, CD86, ICOS, IDO1 and so on. Early studies have reported that sustained T-cell activation induced by a chronic inflammatory environment, for example, during chronic viral infection or in a tumor, caused persistent LAG3 expression on T cells which frequently co-express with other IRs, like PD1, TIM3, TIGIT, CD160, 2B4 and so on, subsequently resulting in a T-cell dysfunction state (73). This state, also named T-cell

functional exhaustion, defined by a distinct subset of exhausted T cells with an elevated expression of IRs, resulting in lack of proliferation, cytokine secretion, and cytolytic activity (15, 74–76).

## 5. Conclusions

The clinical and biological significances of LAG3 were investigated by using sample-sized breast cancer cohorts. Our findings indicated LAG3 was overexpressed in high malignant breast cancer, and was synergistic with other immune checkpoint members. We also depicted the potential profile of LAG3 and immune cell populations, indicating LAG3 might also be involved in B cell immunity. LAG3 expression in cellular components within the tumor microenvironment and the immunoregulatory role of LAG3 need to be addressed in further studies. These findings also lend evidence to combination immunotherapy by targeting LAG3 and other checkpoint members together in breast cancer. This is the largest and most comprehensive study characterizing the LAG3 related transcriptome profile.

### Declarations

## Declarations

### *Ethics approval and consent to participate*

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### *Consent for publication*

Not applicable.

### *Availability of data and material*

The data generated or analyzed during this study are included in this article, or if absent are available from the corresponding author upon reasonable request.

### *Acknowledgments*

The authors thank Pro. Hai Hu and Dr. Jianfang Liu in Chan Soon-Shiong Institute of Molecular Medicine at Windber, for providing us high-quality TCGA breast cancer clinical data. We wish to thank TCGA project organizers as well as all study participants. The first author is particularly indebted to Ping He for help during the study.

### *Funding*

This paper was partially sponsored by grants from the National Natural Science Foundation of China, No. 81872160 (Jing Wang).

## ***Competing interests***

No potential conflicts of interest were disclosed.

## ***Authors Contributions***

Conception and design: Jing Wang (JW), Yi Fang (YF), Qiang Liu (QL). Development of methodology: QL, Yihang Qi (YHQ). Acquisition of data: Jie Zhai (JZ), Xiangyi Kong (XYK), Xiangyu Wang (XYW). Analysis and interpretation of data: QL, JZ. Writing, review and/or revision of the manuscript: QL, YHQ, JZ. Administrative, technical, or material support: XYK, XYW. Study supervision: JW, YF. All authors read and approved the final manuscript.

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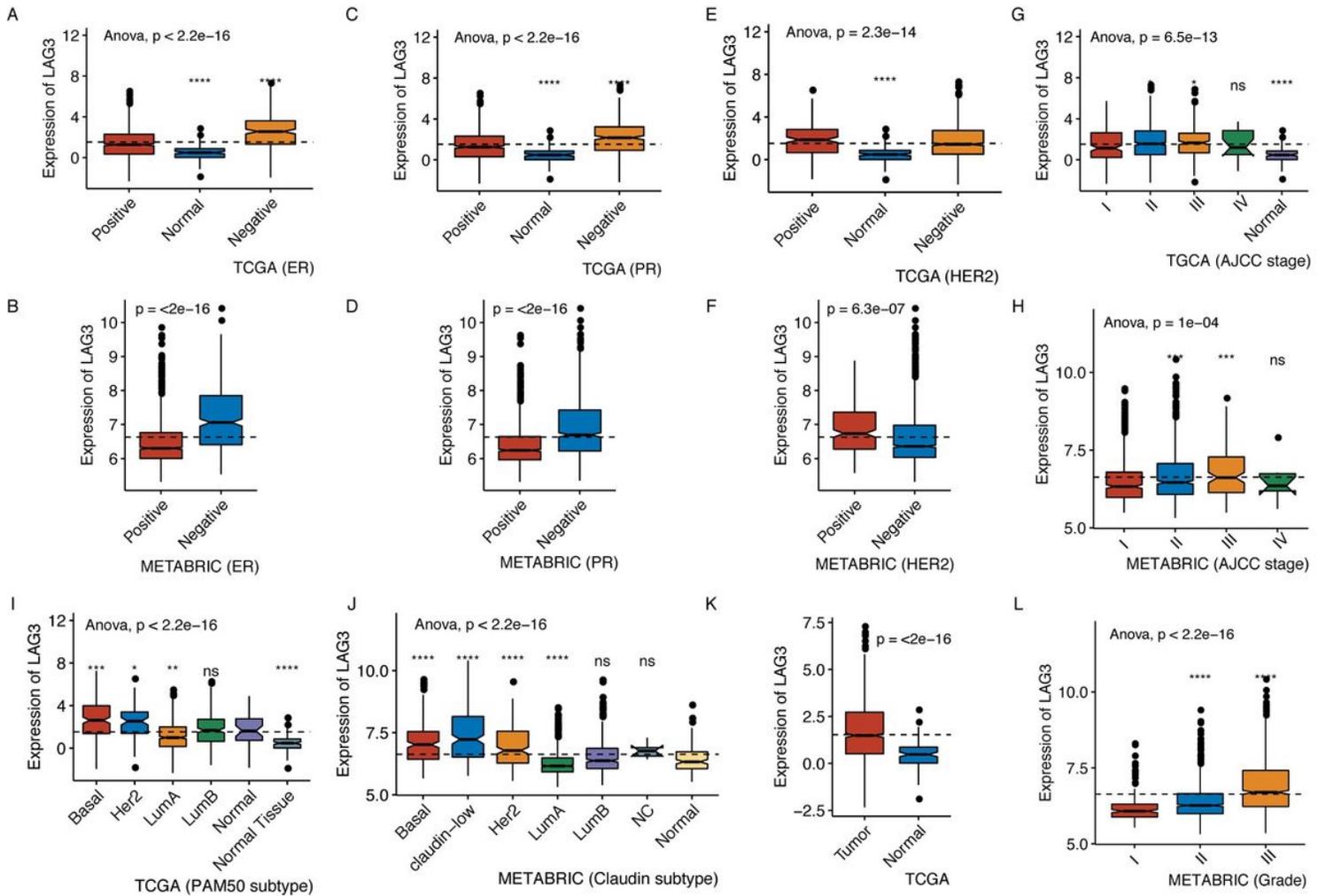
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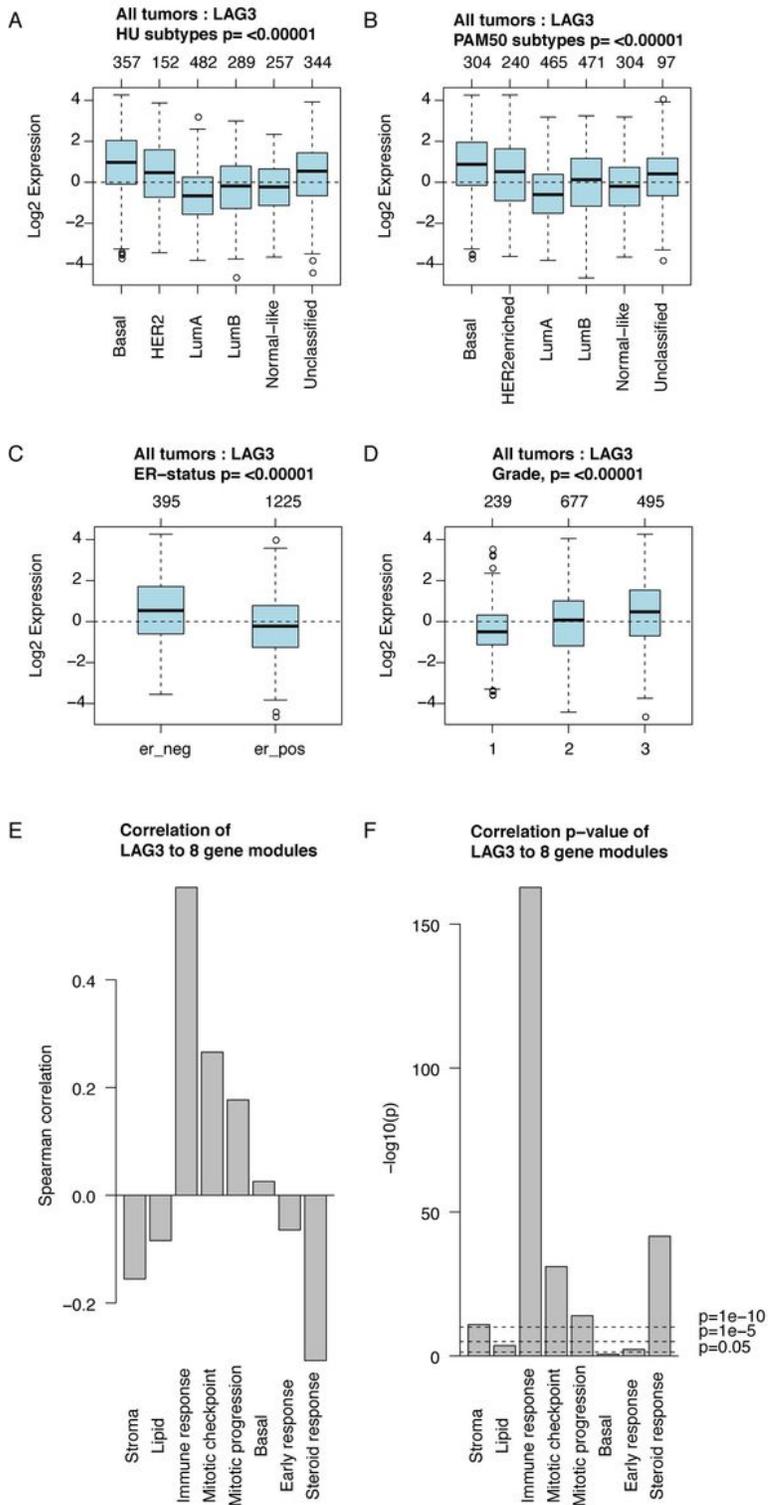
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## Figures



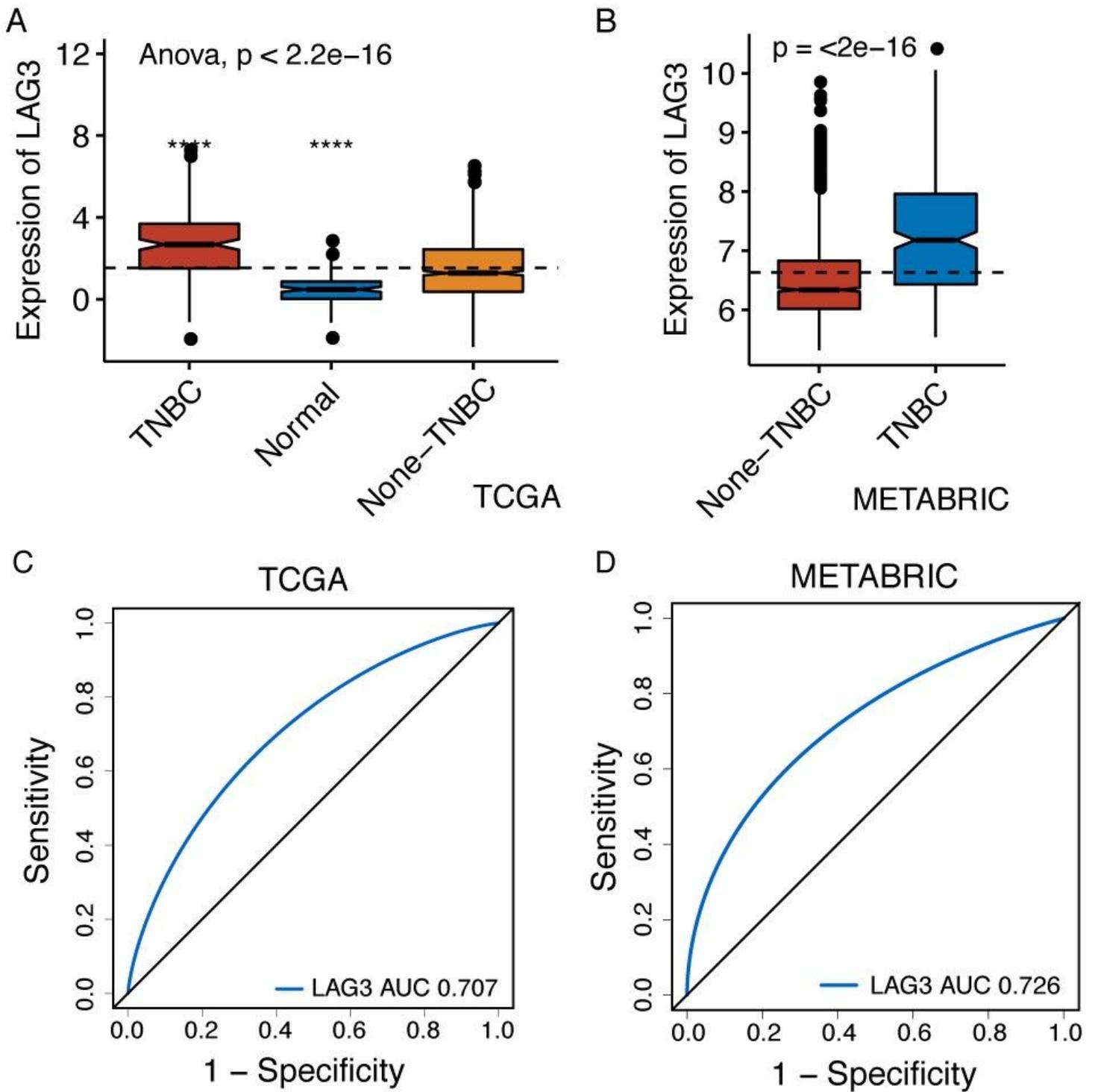
**Figure 1**

LAG3 expression in different molecular subtypes and stage of transcriptional classification scheme in TCGA and METABRIC cohort. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ )



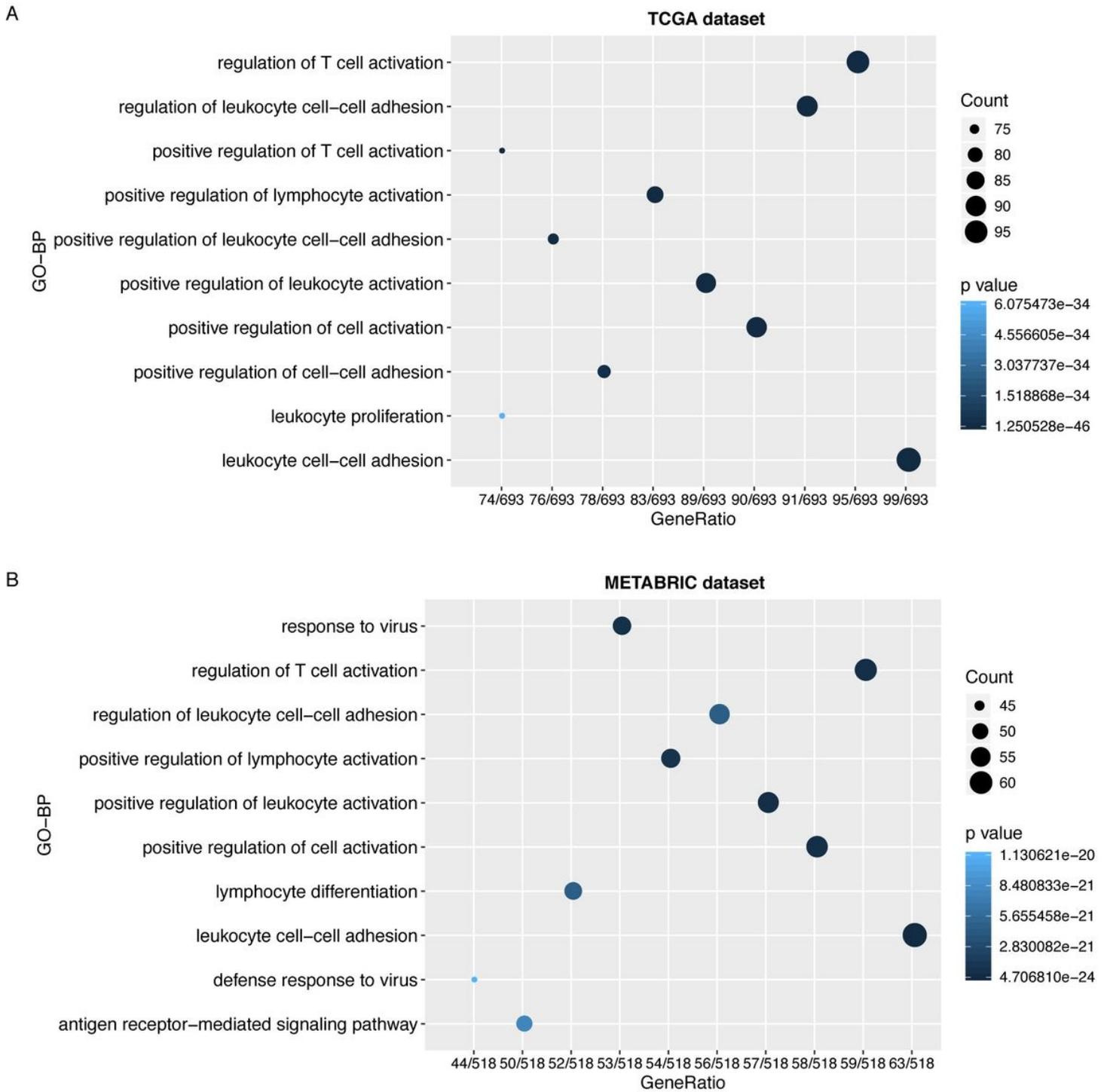
**Figure 2**

LAG3 expression in 1881-sample dataset. LAG expression across different subtypes and tumor stage (A-D); Correlation of LAG3 and gene modules (E and F).



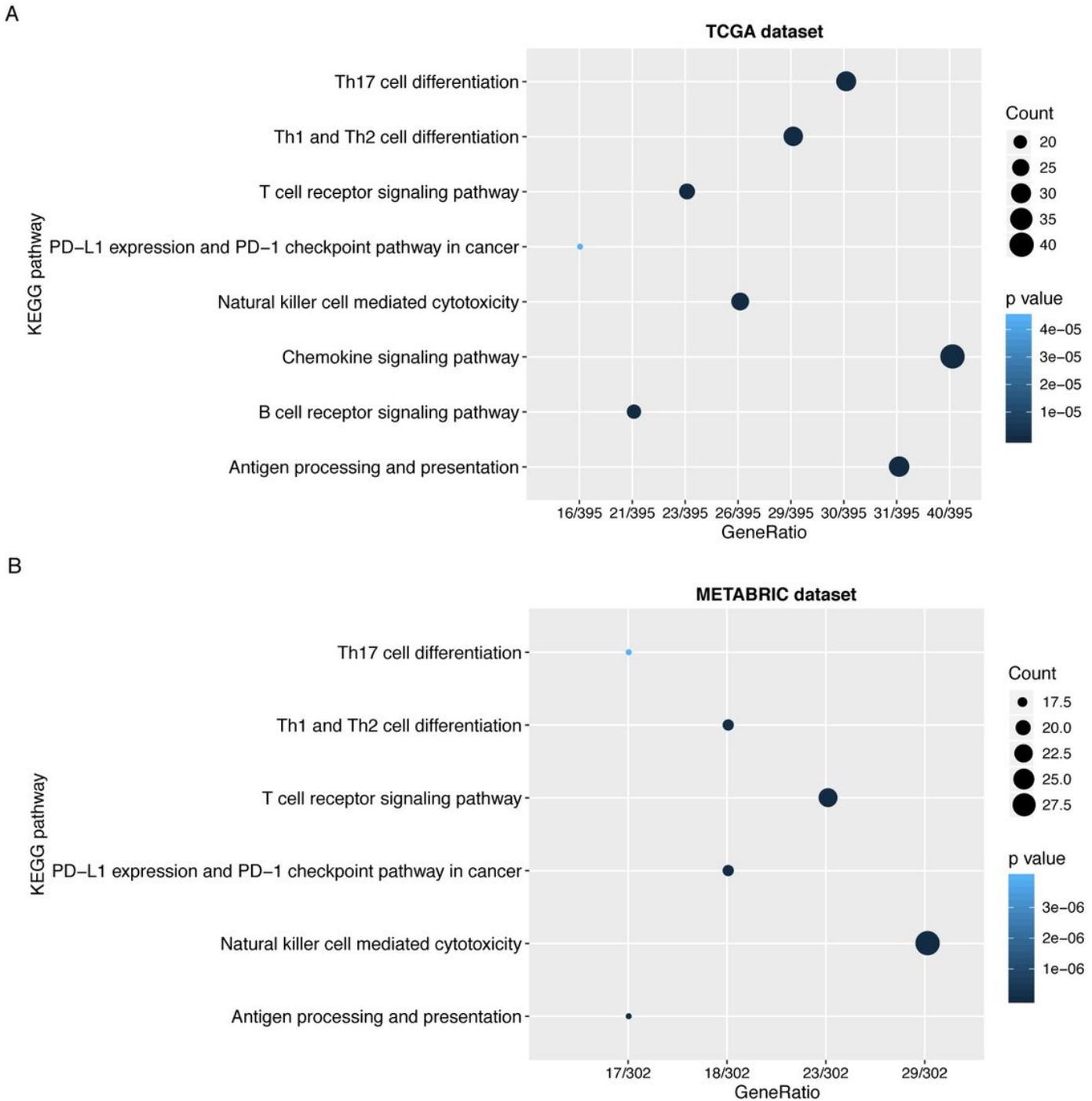
**Figure 3**

LAG3 serves as a potential biomarker. LAG3 expression pattern between TNBC and none-TNBC tissues in TCGA and METABRIC (A and B); ROC curves predicted LAG3 as a biomarker of TNBC (C and D). (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).



**Figure 4**

LAG3 was closely related to immune functions in breast cancer. Gene ontology analysis showed that LAG3 was mainly involved in immune response and inflammatory response in TCGA and METABRIC databases (A and B).



**Figure 5**

LAG3 was closely related to immune cell related signaling pathways. KEGG analysis revealed LAG3 was involved in T cell related signaling pathways, B cell related pathways, and immune checkpoint related pathways (A and B).

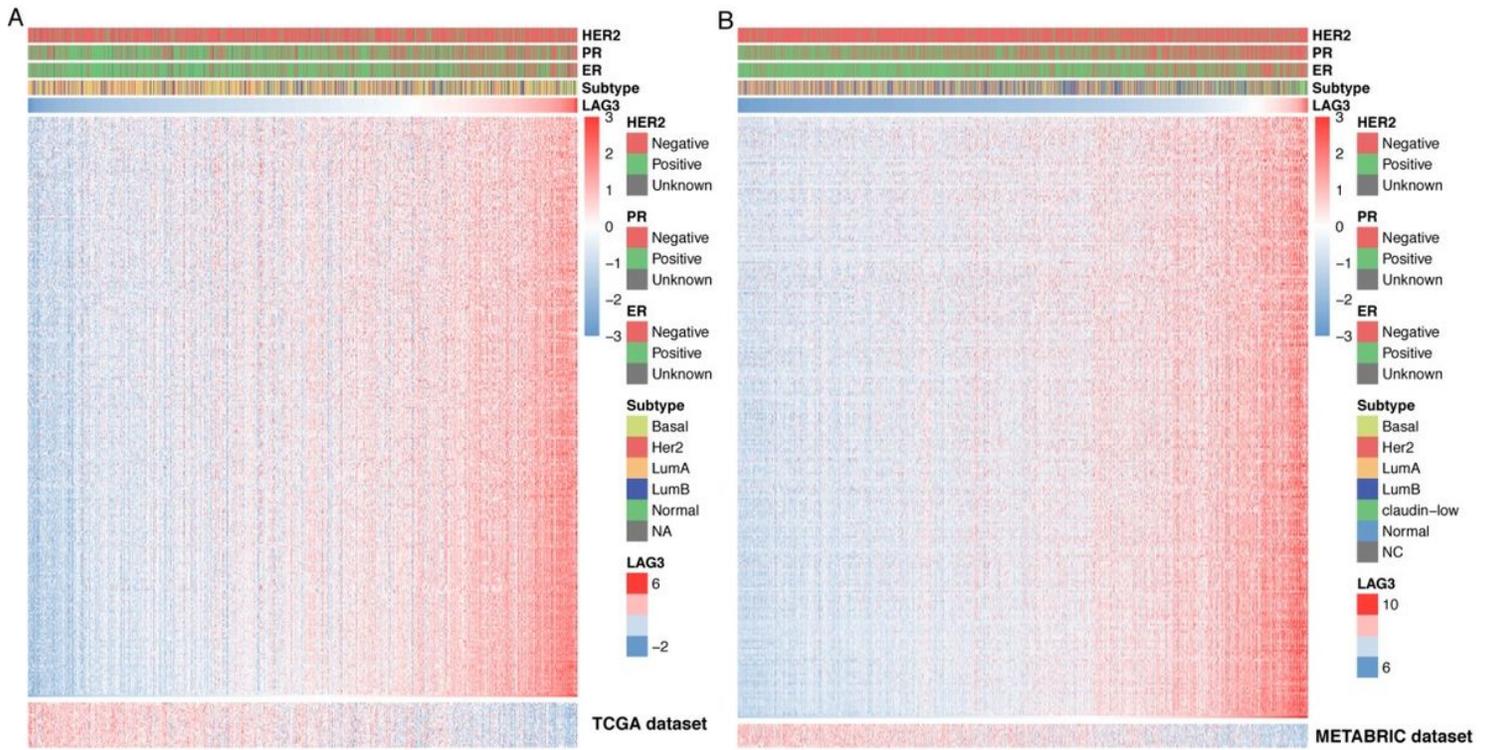


Figure 6

LAG3 related immune responses. Most immune-related genes were positively correlated with LAG3 expression in TCGA and METABRIC databases, while a small number of genes were negatively associated (A and B).

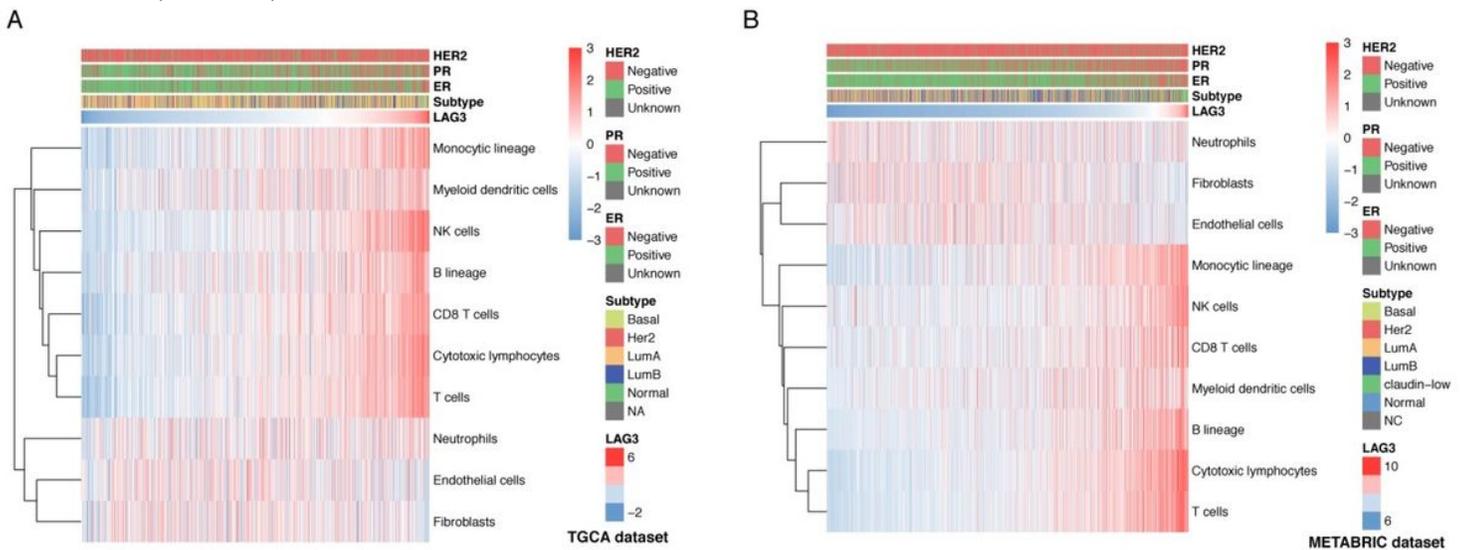
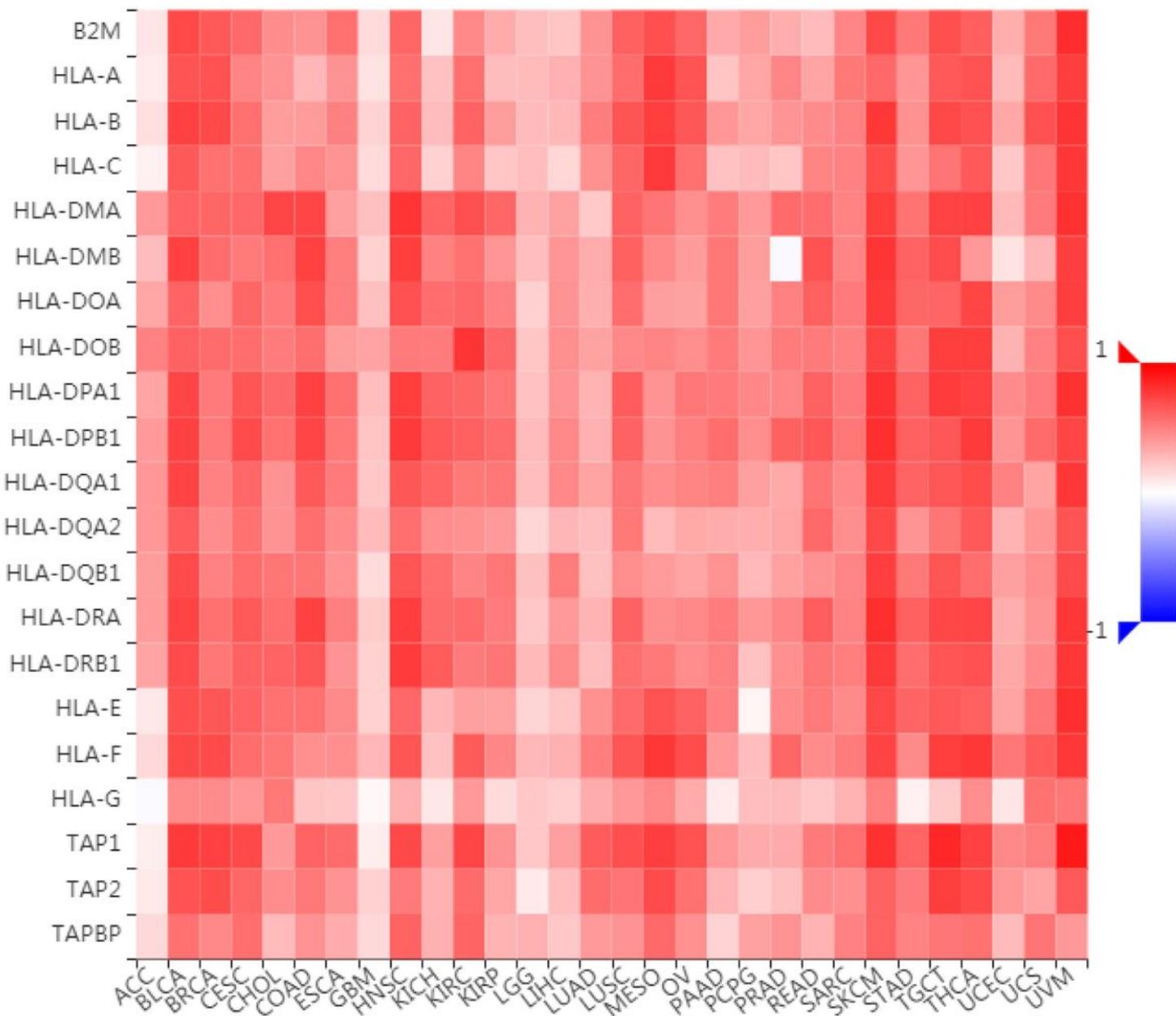


Figure 7

Association between LAG3 expression and immune cell populations in TCGA (A) and METABRIC (B) databases.

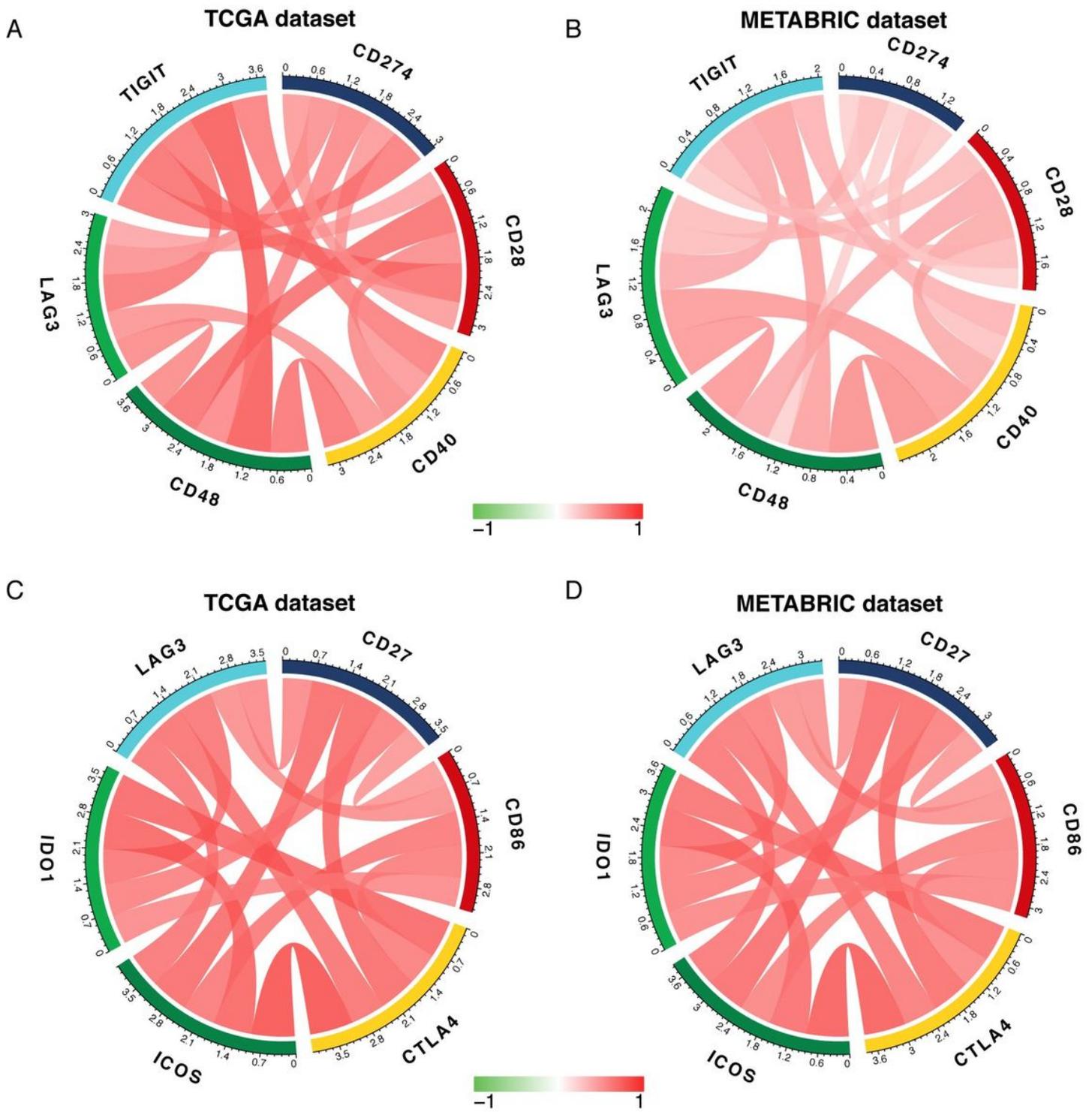






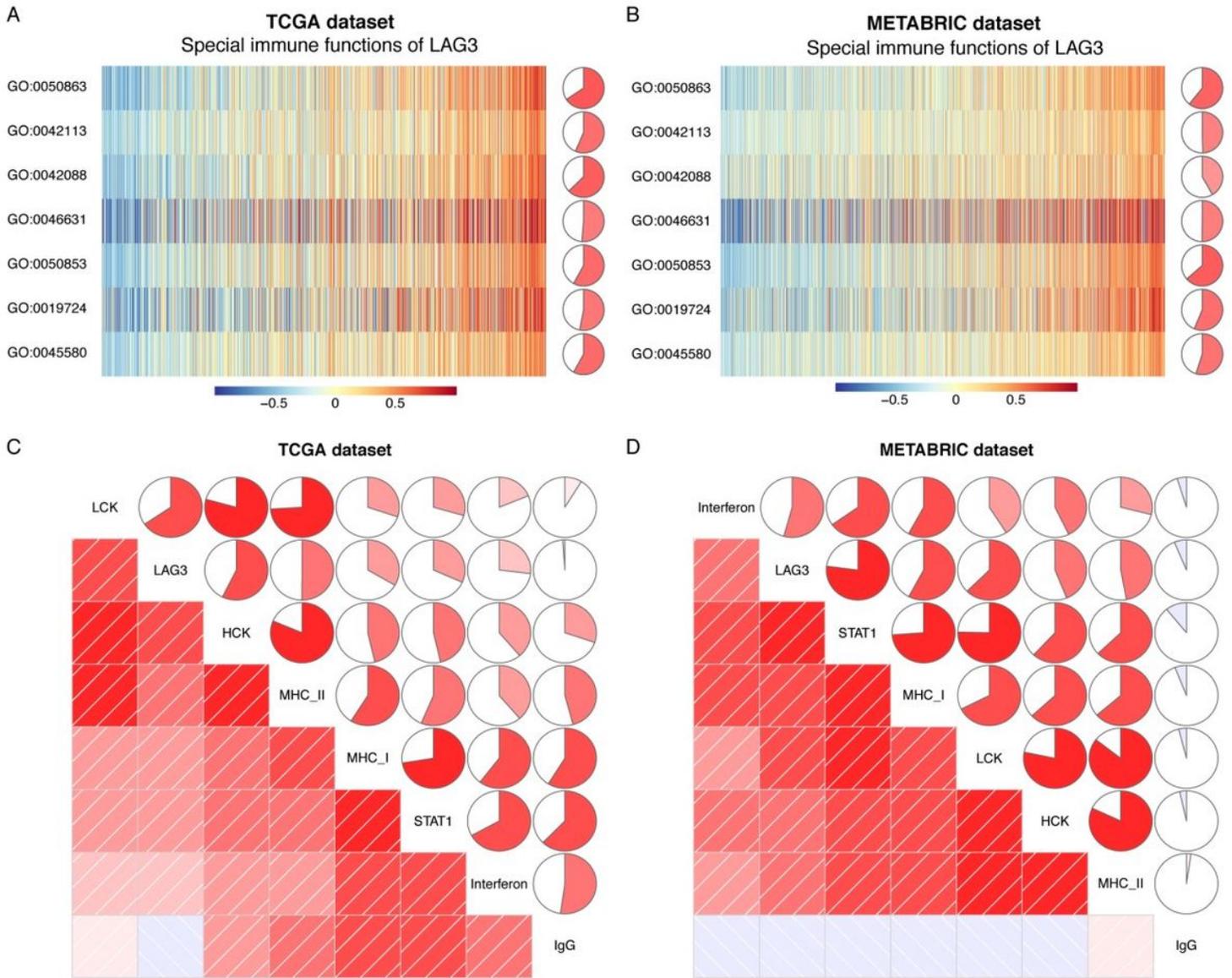
**Figure 10**

LAG3 expression is correlated with MHC molecules in pan-cancer.



**Figure 11**

LAG3 expression is correlated with immune checkpoint members in TCGA and METABRIC databases (A-D).



**Figure 12**

LAG3 related cell immunity and inflammatory activities in breast cancer. The relationship between LAG3 and cell immunity in TCGA and METABRIC datasets (A and B). The relationship between LAG3 and inflammatory activities in TCGA and METABRIC datasets (C and D). GO:0019724: B cell mediated immunity; GO:0042088: T-helper 1 type immune response; GO:0042113: B cell activation; GO:0045580: regulation of T cell differentiation; GO:0046631: alpha-beta T cell activation; GO:0050853 B: cell receptor signaling pathway; GO:0050863: regulation of T cell activation.

## Supplementary Files

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- [CoverLetter.docx](#)

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- TableS1.pdf
- TableS1.pdf