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Original Article

Low expression of INHB co-receptor TGFBR3 in connection with metastasis and immune infiltration in lung adenocarcinoma

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Abstract: Objective: Inhibin B (INHB) is one of the TGF-β superfamily member, consisting of α (INHA) and βB (INHBB) subunits. Studies have found that TGF-β receptor 3 (TGFBR3) binds to a convex α subunit on the surface of INHB, and enhances the binding affinity of activin receptor type-2 (ACVR2A/B) to INHβ subunit. This study tried to evaluate the roles of INHB subunits and its receptors (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) as prognostic biomarkers and therapeutic targets for the effective treatment of lung adenocarcinoma (LUAD). Methods: We analyzed INHB subunits and its receptors' expression and the influence of LUAD from Oncomine, GEPIA, HCMDB, CancerSEA, TIMER databases and so on. Then, 41 cases of cancer tissue and 41 cases of adjacent epithelium were detected in LUAD patients by immunohistochemistry. Results: INHA, ACVR2A, ACVR2B, INHBB were up-regulated while TGFBR3 was down-regulated in LUAD. INHA, ACVR2A and TGFBR3 were found to be strongly associated with high-grade malignancies and advanced TNM, only TGFBR3 expression was negatively correlated with LUAD metastasis probably mainly through cell adhesion molecules and the PI3K-Akt signaling pathway, univariate and multivariate analysis suggested that overall survival was lower in LUAD cases with low TGFBR3 levels. Further analysis revealed that low TGFBR3 expression was related to reduced infiltration of immune cells into the LUAD, promoting metastasis of LUAD cells. TGFBR3 expression negatively correlates with lymphatic metastasis and clinical stage in patients with LUAD. Conclusion: TGFBR3 could be a potential new metastatic biomarker for LUAD, with potential application as a prognostic marker and for immunotherapy of LUAD.

Keywords: Lung adenocarcinoma, inhibin B, co-receptor, TGFBR3, metastasis, immune infiltration

Introduction

Lung cancer is one of the most common cancers (11.6%), and has a high associated mortality rate (18.4%) [1]. At present, 8.2 million lung cancer patients die every year globally, and it is estimated that by 2030, the number of death will rise to 10 million [2, 3]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer incidence, and lung adenocarcinoma (LUAD) is the most prevalent subtype of NSCLC [4]. The diagnosis of LUAD is dependent primarily on imaging and pathological examination, and it is challenging to diagnose lung cancer at its early stage. Furthermore, LUAD is characterized by a high mutational burden [5]. As a result, although various means have been provided for the treatment of LUAD, such as radiotherapy, surgery, chemotherapy, immunotherapy, and targeted molecular drugs, the prognosis is still unsatisfactory and the fiveyear survival rate is low [6, 7]. Therefore, it is critical to find out helpful prognostic biomarkers and therapeutic targets for the effective treatment of LUAD.

Inhibin (INH) is one of the transforming growth factor-β (TGF-β) superfamily, consisting of α and β (βA and βB) subunits, with isomers of the two subunits forming inhibin A (α and βA) and inhibin B [α (INHA) and βB (INHBB)], respectively. INHB is mainly secreted by germ cells and acts as a negative feedback regulator of Follicle-stimulating hormone (FSH). Activin (ACT), another of the TGF-β superfamily, is a homodimer composed of β subunits with 63% amino acid homology to the mature growth factor region of the INHβ subunit [8]. INH can antago-

nize ACT signal transduction for biological function. INH binds to the activin receptor type-2 (ACVR2) and competitively inhibits the ACT signaling pathway [9].

Subunit β binds specifically to ACVR2. However, it was found that ACVR2 has a 10-fold lower INH binding affinity than ACT and fails to stimulate intracellular signaling. Therefore, higher concentrations of INH are required to inhibit ACT activity through theoretical receptor antagonism [10, 11]. However, it was found that INH can compete with ACT signaling at the same concentration of ACT molecules, suggesting that INH may have higher affinity for binding proteins or co-receptors for cell-specific signaling [10]. Chapman SC et al. found that transforming growth factor β receptor 3 (TGFBR3) binds to a convex α subunit on the surface of INH and enhances the binding affinity of ACVR2A/B to INHβ subunit [12]. So, INH-ACVR2A/B-TGFBR3 forms a high-affinity ternary complex that antagonizes ACT signal transduction.

The research of INHB on oncology is limited mainly to reproductive system tumors. Our previous research has found that INH βB inhibited invasion and metastasis of nasopharyngeal carcinoma cells through the TGF-β/Smads signaling pathway [13]. As to the mechanism, we found that INHB subunits and INHB receptors are differentially expressed in lung cancer. In this study, we intend to analyze the effect of INHB on lung cancer through bioinformatics in this manuscript.

Materials and methods

Oncomine analysis

The mRNA expression of INHB was analyzed in the Oncomine database (http://www.oncomine.org) [14], including the transcription levels of INHB α subunit (INHA) and βB subunit (INHBB) and receptors (ACVR2A, ACVR2B and TGFBR3) in LUAD were compare with those in normal controls. Fold change >1.5 with *P* values <0.01 was considered statistically significant.

UALCAN analysis

The UALCAN platform (http://ualcan.path.uab.edu) was used to depict the expression profile for INHB and its receptors (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) in LUAD patients and explore expression profile of these proteins based on clinicopathologic factors [15]. The GEPIA platform (http://gepia.cancer-pku.cn) was performed for analyzing the RNA sequencing expression of INHB and its receptors from the TCGA and the GTEx projects with a standard processing pipeline, including according to pathological stages [16].

The Kaplan-Meier plotter analysis

The prognostic significance of INHB (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) in LUAD was assessed by the Kaplan-Meier plotter (https://kmplot.com/analysis) [17], shortlisted patients were carved up high and low groups based on expression the gene, we assessed the association with overall survival (OS), first progression (FP), post progression survival (PPS) in LUAD patients at risk values by R software package (version 3.6.3) [18], which was denoted by log rank *P*-value and hazarded ratio (HR) with 95% confidence intervals.

Cancer SEA analysis

To further understand the function of these genes, we used the Cancer SEA, an online database comprehensively decoding distinct functional states of cancer cells at single-cell resolution (http://biocc.hrbmu.edu.cn/CancerSEA) [19], to analyze the correlations between INHB (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) and functional states in different single-cell datasets.

HCMDB analysis

To understand the relationship between INHB and LUAD metastasis, HCMDB, an online database designed to store and analyze the large-scale expression of cancer metastasis (http://Human Cancer Metastasis Database) [20], was examined the expression of metastasis-associated genes. We analyzed the expression of INHB (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) in LUAD metastasis samples of GSE1987 and the co-expression of the genes.

PPI networks analysis

The STRING database (https://string-db.org, version 11.0) was used for analysis of the molecular interactions between TGFBR3 and co-expression genes from HCMDB database (The confidence score >0.4 was considered

statistically significant) [21]. Then, we performed Cytoscape (version 3.8.2) to visualize the molecular interaction networks [22].

GO/KEGG pathway enrichment analysis

The R software package performed GO/KEGG enrichment analyse of TGFBR3 and co-expression genes (version 3.6.3) [18]. $P<0.05$ was considered statistically significant for GO annotation enrichment analysis and KEGG pathway enrichment analysis.

TIMER database analysis

The correlation of TGFBR3 expression with immune infiltration was determined using the TIMER (http://timer.cistrome.org/), we analyzed the abundance of six types of infiltrating immune cells (CD8+ T cells, CD4+ T cells, B cells, neutrophils, macrophages and myeloid dendritic cells), and the correlation between TGFBR3 expression and several immune cell markers to determine potential immune cell subtypes infiltrating patients with LUAD.

Validation of TGFBR3 function in LUAD metastasis

All the samples were obtained from September 2019 to January 2021 at the Department of Oncology, the Second People's Hospital of Hunan Province (Changsha, China). The study was approved by our institution's ethics committee, and all participants gave informed consent before inclusion. The patients' mean age was 61 years (range, 48-76 years). The samples included 41 cases of cancer and non-cancerous tissues of LUAD patients. Immunohistochemistry was carried out using a standard procedure as previously described. The results were determined by staining intensity and the number of positive cells [13]. The product of the two scores was taken as the total score ≥4 was considered a high expression, while <4 was considered a low one.

Statistical analysis

Statistical analysis was analysed by SPSS25.0 software. Relationship between TGFBR3 expression and clinicopathology, characteristics of LUAD patients were analyzed through Pearson χ^2 and continuity correction χ^2. In the data analysis of the database used in this manuscript, if homogeneity of variance test, T test was used to compare INHB and its receptors transcription levels between lung adenocarcinoma and normal tissues, if heterogeneity of variance test, Mann-Whitney U test was selected. The expression difference of INHB and its receptors among different clinical characteristics was tested by One-Way analysis of variance (Kruskal-Wallis Test). DeLong's test for the ROC curve of INHB and its receptors for LUAD. Kaplan-Meier method was used for survival analysis in patients with LUAD. Univariate and multivariate regression analysis for TGFBR3 and clinicopathologic parameters with OS, FP and PPS in LUAD patients. Spearman's correlation analysis for correlations between TGFBR3 and gene markers of immune cells.

Results

INHB and its receptors expression in LUAD patients

We analyzed the INHB (including INHA and INHBB) expressions and its receptors (including ACVR2A, ACVR2B, and TGFBR3) in lung cancer patients from the Oncomine database. Compared with normal lung tissue, there were no variation in INHA expression and high expression levels of INHBB, ACVR2A and ACVR2B compared to normal lung tissues, and ACVR2B expression was increased, while TGFBR3 expression was decreased in cancer tissues compared to lung cancer patients (**Figure 1A**; **Table 1**). There were eight significant datasets for the low expression of TGFBR3 ($P<0.001$) and one significant dataset for the high expression of INHBB ($P<0.001$) from the Oncomine database (**Figure 1B-J**).

The relationship of INHB and its receptors' expression with clinicopathologic characteristics in LUAD patients

To improve the reliability of the results of the above bioinformatic analysis, we further analyzed the INHB levels and clinical data in TCGA (INHA, ACVR2A, ACVR2B, INHBB, TGFBR3) for 513 patients with the clinical characteristics shown in **Table 2**; unpaired comparisons tumors and patients in the INHB and UALCAN databases, the expression levels of INHA, INHBB and ACVR2B were elevated ($P<0.001$, <0.01, <0.001, respectively), while the expression levels of ACVR2A and TGFBR3 were downregulated ($P<0.05$, <0.001, respectively) in LUAD patients (**Figure 2A**). Paired comparison of INHB and its receptors' expression in tu-

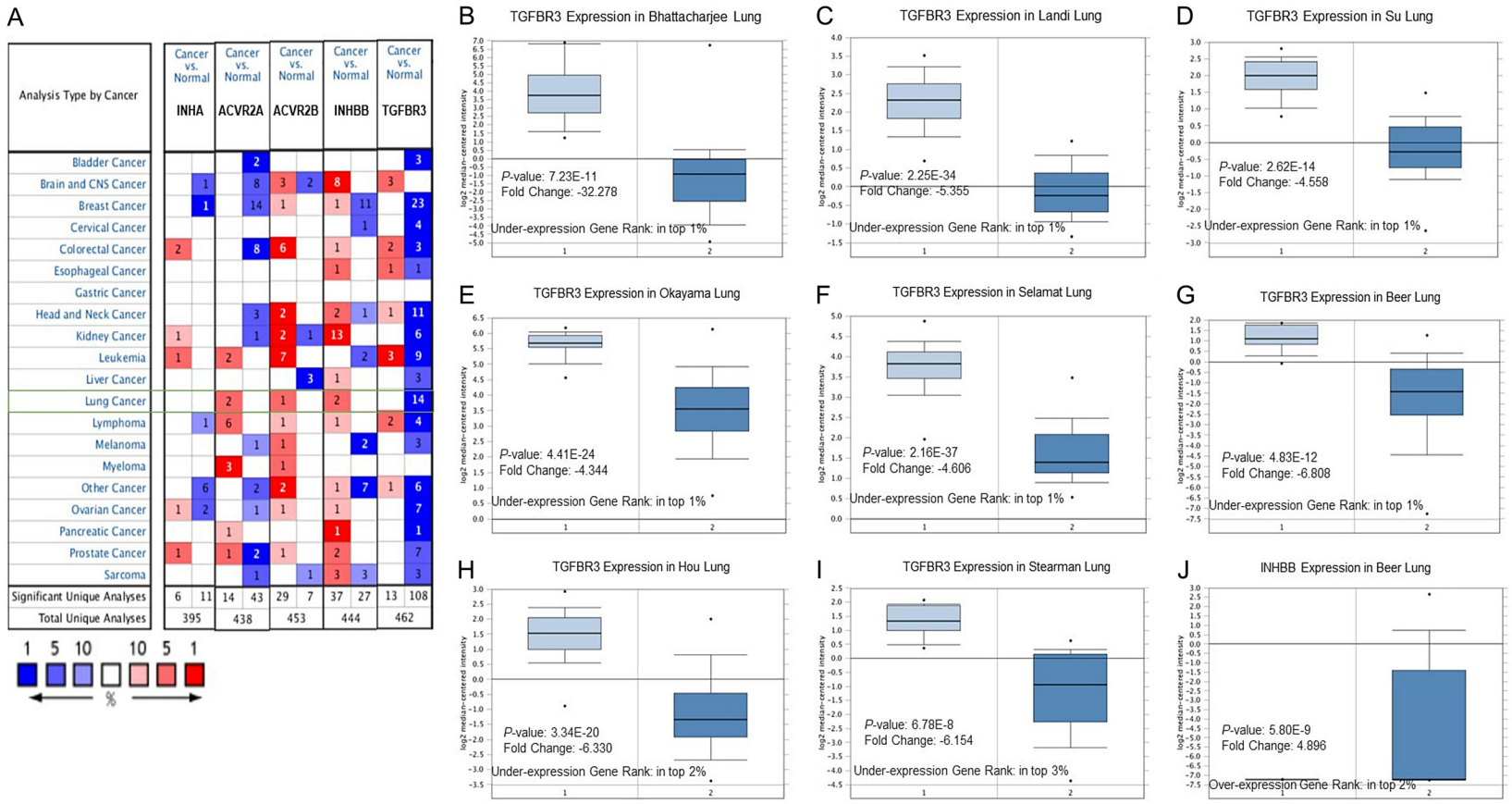

Figure 1. Inhibin B and its receptors expression in LUAD patients (Oncomine). A. Red represents up-regulation and blue represents down-regulation in the tumor tissues. The color is determined by the best gene rank percentile for the analyses. The number within cells means the number of datasets. B-I. The expression of TGFBR3 in LUSD patients in eight datasets. J. The expression of INHBB in LUSD patients in one datasets. (INHA: Inhibin α subunit; INHBB: Inhibin βB; ACVR2A: activin receptor type-2A; ACVR2B: activin receptor type-2B; TGFBR3: transforming growth factor β recetor 3; LUAD: lung adenocarcinoma).

Table 1. INHBB/TGFBR3 Significant Changes in Transcription Levels in Lung Adenocarcinoma and Normal Tissue (Oncomine)

	Cancer and Normal Type	Fold Change	*P*-value	t-Test	Reference Data
INHBB	Lung Adenocarcinoma	4.896	5.80E-9	6.320	Beer Lung (96)
TGFBR3	Lung Adenocarcinoma	-32.278	7.231E-11	-11.303	Bhattacharjee Lung (149)
	Lung Adenocarcinoma	-5.355	2.25E-34	-18.633	Landi Lung (107)
	Lung Adenocarcinoma	-4.558	2.62E-14	-10.829	Su Lung (57)
	Lung Adenocarcinoma	-4.344	4.41E-24	-18.553	Okayama Lung (246)
	Lung Adenocarcinoma	-4.606	2.16E-37	-19.117	Selamat Lung (116)
	Lung Adenocarcinoma	-6.808	4.83E-12	-9.541	Beer Lung (96)
	Lung Adenocarcinoma	-6.330	3.34E-20	-12.985	Hou Lung (110)
	Lung Adenocarcinoma	-6.154	6.78E-8	-7.451	Stearman Lung (39)

Note: INHBB: inhibin βB subunit; TGFBR3: transforming growth factor β recetor 3.

Table 2. Clinical Characteristics of the Lung Adenocarcinoma Patients (TCGA)

Characteristic		N	%
Gender	Female	276	53.8
	Male	237	46.2
Age	≤65	238	48.2
	>65	256	51.8
Race	Asian	7	1.6
	Black or African American	54	12.0
	White	387	86.4
Pathologic stage	Stage I	274	54.3
	Stage II	121	24.0
	Stage III	84	16.6
	Stage IV	26	5.1
T stage	T1	168	33.0
	T2	276	54.1
	T3	47	9.2
	T4	19	3.7
N stage	N0	330	65.9
	N1	95	19.0
	N2	74	14.7
	N3	2	0.4
M stage	M0	344	93.2
	M1	25	6.8
Smoker	No	74	14.8
	Yes	425	85.2
Number pack years smoked	<40	174	49.6
	≥40	177	50.4
Anatomic neoplasm subdivision	Central Lung	62	32.8
	Peripheral Lung	127	67.2
Primary therapy outcome	PD+SD	105	24.6
	PR+CR	321	75.4

Note: PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response.

mors and regular patients from the UALCAN database, the expression of ACVR2A had no difference, the expression levels of INHA, INHBB, and ACVR2B were up-regulated (*P*<0.001, <0.01, <0.01, respectively). In contrast, the expression TGFBR3 were down-regulated (*P*<0.001) in LUAD patients (**Figure 2B**).

The TCGA database found INHB and its receptors to distinguish LUAD from normal. ROC curve analysis showed that the area under the curve (AUC) of INHA, ACVR2A, ACVR2B, INHBB, and TGFBR3 in diagnosing LUAD were 0.820, 0.548, 0.724, 0.646, and 0.970, respectively. TGFBR3 had higher specificity (91.2%), sensitivity (91.5%) and diagnostic efficiency (**Figure 2C**; **Table 3**).

We further found that in LUAD patients, INHA, ACVR2A, and TGFBR3 were significantly correlated with pathological stage from the GEPIA database (**Figure 2D-H**). Also, INHA, ACVR2A, and TGFBR3 were significantly correlated with lymphatic metastasis from the UALCAN database (**Figure 2I-M**), while ACVR2B

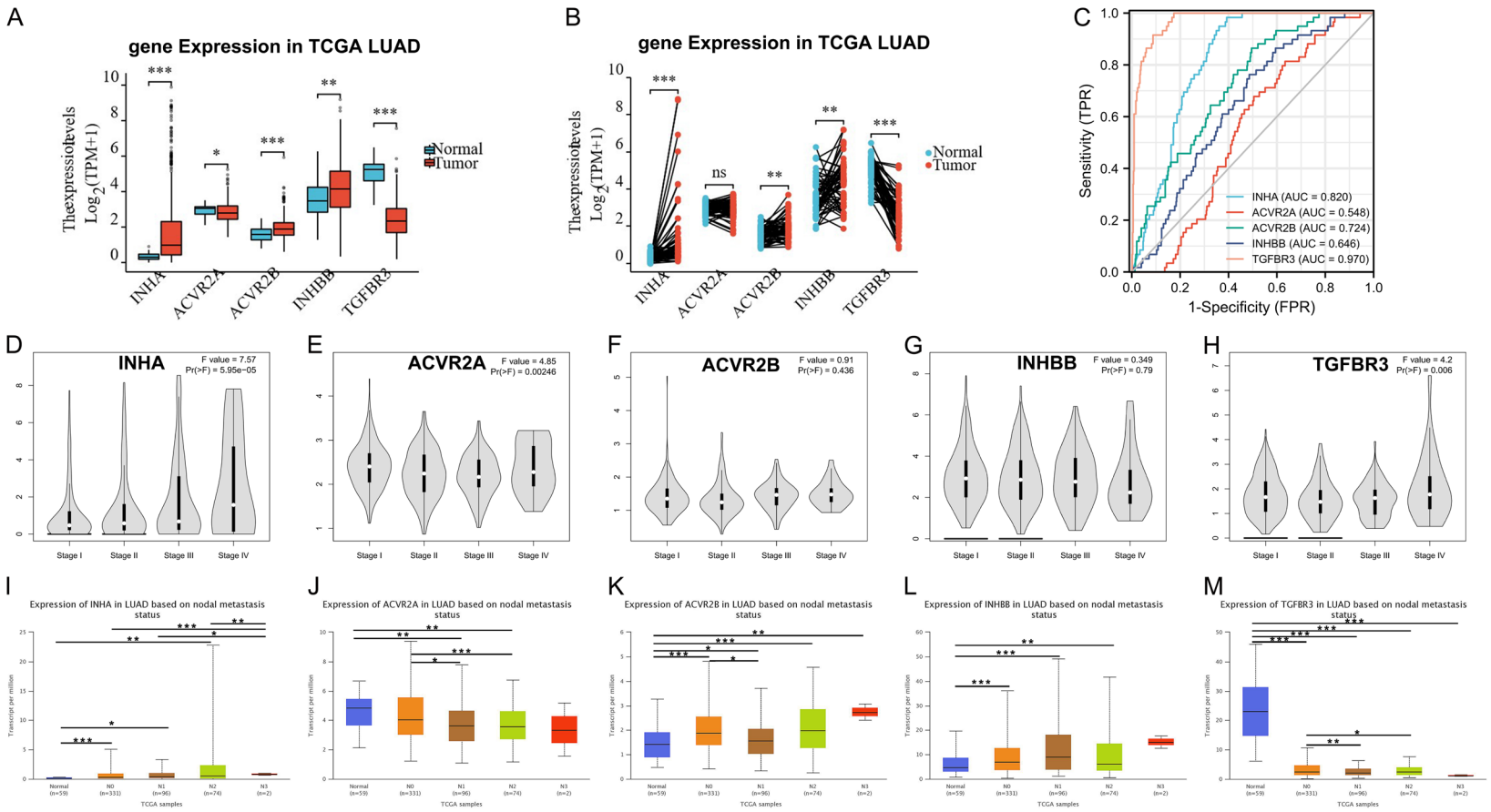

Figure 2. Inhibin B and its receptors expression of patients with LUAD according to different clinical characteristics (UALCAN and GEPIA). A. Unpaired comparison of INHB and its receptors expression in tumors and normal patients from the UALCAN; B. Paired comparison of INHB and its receptors expression in tumors and normal patients from the UALCAN; C. The ROC curve of INHB and its receptors for LUAD. D-H. INHB and its receptors were significantly correlated with pathological stage from the GEPIA; I-M. INHB and its receptors were significantly correlated with lymphatic metastasis from the UALCAN.

Table 3. Diagnostic Efficiency of INHB and its receptors for LUAD (TCGA)

Predictor	Cut-off Value	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Youden Index
INHA	0.260	0.607	0.983	0.997	0.216	1.591
ACVR2A	1.431	0.366	0.814	0.947	0.124	1.180
ACVR2B	0.911	0.505	0.864	0.971	0.161	1.369
INHBB	2.651	0.514	0.763	0.952	0.148	1.277
TGFBR3	2.455	0.912	0.915	0.990	0.535	1.827

Note: INHA: inhibin α subunit; ACVR2A: activin receptor type-2A; ACVR2B: activin receptor 2B; INHBB: inhibin βB subunit; TGFBR3: transforming growth factor β recetor 3.

and INHBB had did not correlate with pathological stage and lymphatic metastasis (**Figure 2D-M**).

The association of INHB and the expression of its receptors with prognosis in LUAD patients

Since INHB and its receptors were associated with pathological stage and lymphatic metastasis of LUAD patients, we continued to analyze whether these genes were associated with prognosis using Kaplan-Meier plots. The results demonstrated that the expression of INHA, ACVR2A, ACVR2B, and TGFBR3 were significantly correlated with OS prognosis, while the expression of INHBB was not correlated (**Figure 3A-E**), the LUAD patients with low expression of INHA and ACVR2B, High expression of TGFBR3 and ACVR2A had a better prognosis of OS (P<0.001, 0.014, <0.001, <0.001, respectively). The expression of INHA, ACVR2A, and TGFBR3 were significantly associated with the prognosis of FP. At the same time, ACVR2B and INHBB were not associated (**Figure 3F-J**), the LUAD patients with low expression of INHA, High expression of TGFBR3 and ACVR2A had a better prognosis of FP (P<0.001). Only INHA expression was strongly associated with the prognosis of PPS, while the expression of the other four genes was not associated (**Figure 3K-O**), the LUAD patients with low expression of INHA had a better prognosis of PPS (P=0.0034).

The functional relevance of scRNA-seq of INHB and its receptors in LUAD

The above analysis could be concluded that among INHB and its receptors, INHA, ACVR2B and TGFBR3 were associated with lymphatic metastasis and prognosis of LUAD patients. We investigated further and found out the levels of ACVR2A and TGFBR3 expression had a significant correlation between angiogenesis and metastasis among 14 functional states using the single-cell sequencing database, Cancer SEA. In contrast, INHA had no functional relevance in LUAD (**Figure 4**).

Validation of the relationship between TGFBR3 and lung adenocarcinoma metastasis

We further analyzed the correlation between ACVR2A, TGFBR3 and the metastasis of LUAD (**Figure 5A-E**). In the cancer metastasis database, HCMDB, we located the GSE1987 dataset about the metastasis of LUAD. Only TGFBR3 had differential expression (P<0.001). Based on the results of above database, we choosing TGFBR3 for further bioinformatics analysis.

The major genes co-expressed with the TGFBR3 gene in LUAD were checked using GSE1987, and protein-protein interaction (PPI) networks were generated in the STRING protein interaction database and imported into the Cytoscape bioinformatics software platform (Version 3.8.2) for visualization (**Figure 5F**) and further analysis, glypican 3 (GPC3) and fibroblast growth factor receptor 1 (FGFR1) were considered to be important proteins in contact with TGFBR3 molecule in LUAD. The results of functional annotation enrichment analysis demonstrated that the co-expressed genes were considerably enriched in biological processes in particular positive regulation of phosphatidylinositol 3-kinase signaling, response to corticosteroid and muscle organ development, molecular functions such as extracellular matrix structural constituent, glycosaminoglycan binding and heparin binding, and cellular components such as external side of plasma membrane, cortical cytoskeleton and collagen-containing extracellular matrix. The enriched KEGG pathways included PI3K-Akt signaling, regulates lipolysis and cell adhesion molecules in adipocytes (**Figure 5G**).

Lower TGFBR3 mRNA expression showing shorter OS and FP in LUAD patients

Next, we probed the potential connection of TGFBR3 expression in LUAD. By incorporating

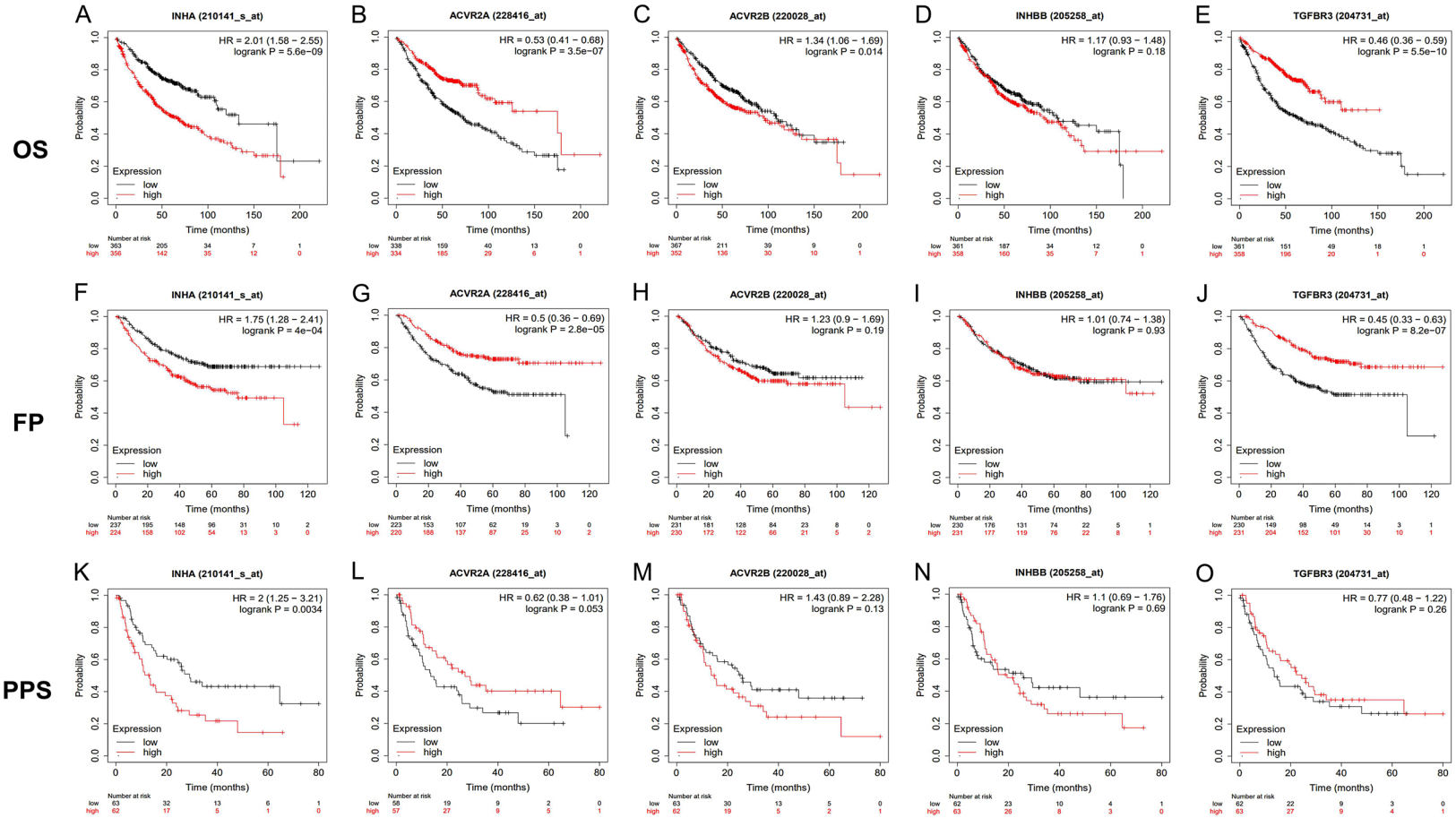

Figure 3. The association of Inhibin B and its receptors expression with prognosis in LUAD patients (Kaplan-Meier plots). A-E. The expression of INHA, ACVR2A, ACVR2B and TGFBR3 were significantly associated with the prognosis of overall survival while INHBB expression was no association. F-J. The expression of INHA, ACVR2A and TGFBR3 were significantly connected with the prognosis of first progression while ACVR2B and INHBB were no connection. K-O. Only INHA expression was substantially related to the prognosis of post progression survival while the expression of other four genes were no relation. OS: overall survival; FP: first progression; PPS: post-progressive survival.

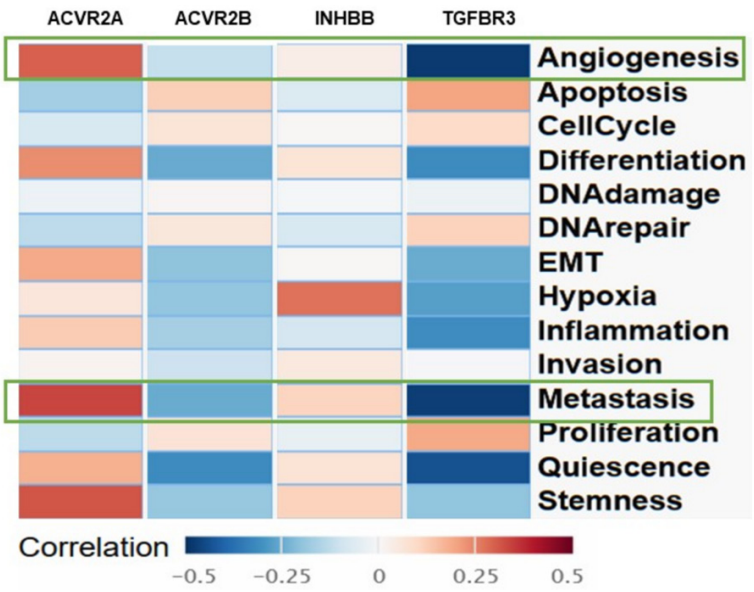

Figure 4. The functional relevance of INHB and its receptors in LUAD patients (CancerSEA). INHA had no functional relevance in LUAD patients, there were the significant correlation of angiogenesis metastasis in ACVR2A and TGFBR3 across 14 functional states. Red represents positive correlation and blue represents negative correlation in the Heat map. The color is determined by the rank of relevance.

clinical and pathological data into a Kaplan-Meier plot, we investigated the relationship between TGFBR3 expression and clinicopathological features in LUAD patients. According to the univariate Cox model, both low TGFBR3 expression and high phase were negative predictive factors for LUAD patients (P<0.001, P=0.039, respectively) (**Figure 6A**). For FP, low TGFBR3 expression was a negative predictor in LUAD patients (P<0.001) (**Figure 6B**). Also, gender and smoking history correlated with OS and FP (P<0.05). In the multivariate Cox model, TGFBR3 expression was an independent factor correlated with OS (P=0.0232) and FP (P=0.0105) (**Figure 6D**, **6E**). TGFBR3 expression and clinicopathologic characteristics had no correlation with PPS in LUAD patients, both univariate and multivariate Cox models (**Figure 6C**, **6F**).

Correlation of TGFBR3 expression with immune infiltration level in LUAD

Immune cells in the tumor microenvironment influence the survival of cancer patients, and previous analysis showed that lower TGFbR3 expression was suggestive of tumor metastasis in LUAD. TGFBR3 expression is suggestive of tumor metastasis in LUAD. Therefore, Exploring the immune infiltration and TGFBR3 expression would be significant. We analyzed whether TGFBR3 expression correlated with the infiltrating immune cells and markers in LUAD by counting the coefficient of TGFBR3 expression and infiltrating immune cells and markers in TIMER.

The results suggested that TGFBR3 expression had negative connected with tumor purity in LUAD (Rho= -0.239, P=7.38e-08), while TGFBR3 expression had positive correlations with CD8+ T cells (Rho=0.169, P=1.64e-04), CD4+ T cells (Rho=0.205, P=4.25e-06), neutrophils (Rho=0.184, P=3.94e-05), macrophages (Rho=0.28, P=2.49e-10), and dendritic cells (Rho=0.176, P=8.35e-05) in LUAD, but no association with B cells (Rho=0.029, P=0.520) (**Figure 7**).

With the purpose of further investigate the potential role of TGFBR3 in the infiltration of immune cells in LUAD, we performed the correlation between TGFBR3 expression and several immune cell markers using the TIMER database, such as B cells, CD8+ T cells, T cells, M1/M2 macrophages, tumor-associated macrophages (TAM), monocytes, neutrophils, NK (natural killer cell) and DC. After adjusting for tumor purity, the TGFBR3 expression level was significantly correlated with 36 out of 52 immune cell markers in LUAD. We found a high correlation of expression with DC cells, but not with CD8+ T cells, T cell depletion and NK cells. There was no correlation with CD8+ T-cell markers, T-cell depletion and NK cells (**Table 4**).

Validation of TGFBR3 expression with metastasis in LUAD patients

We performed immunohistochemistry on cancer tissues from 41 LUAD patients and 41 adjacent epithelial tissues and showed that TGFBR3 positive signals in pulmonary gland epithelial tissues, mainly localized in the cell membrane and cytoplasm (**Figure 8**). TGFBR3

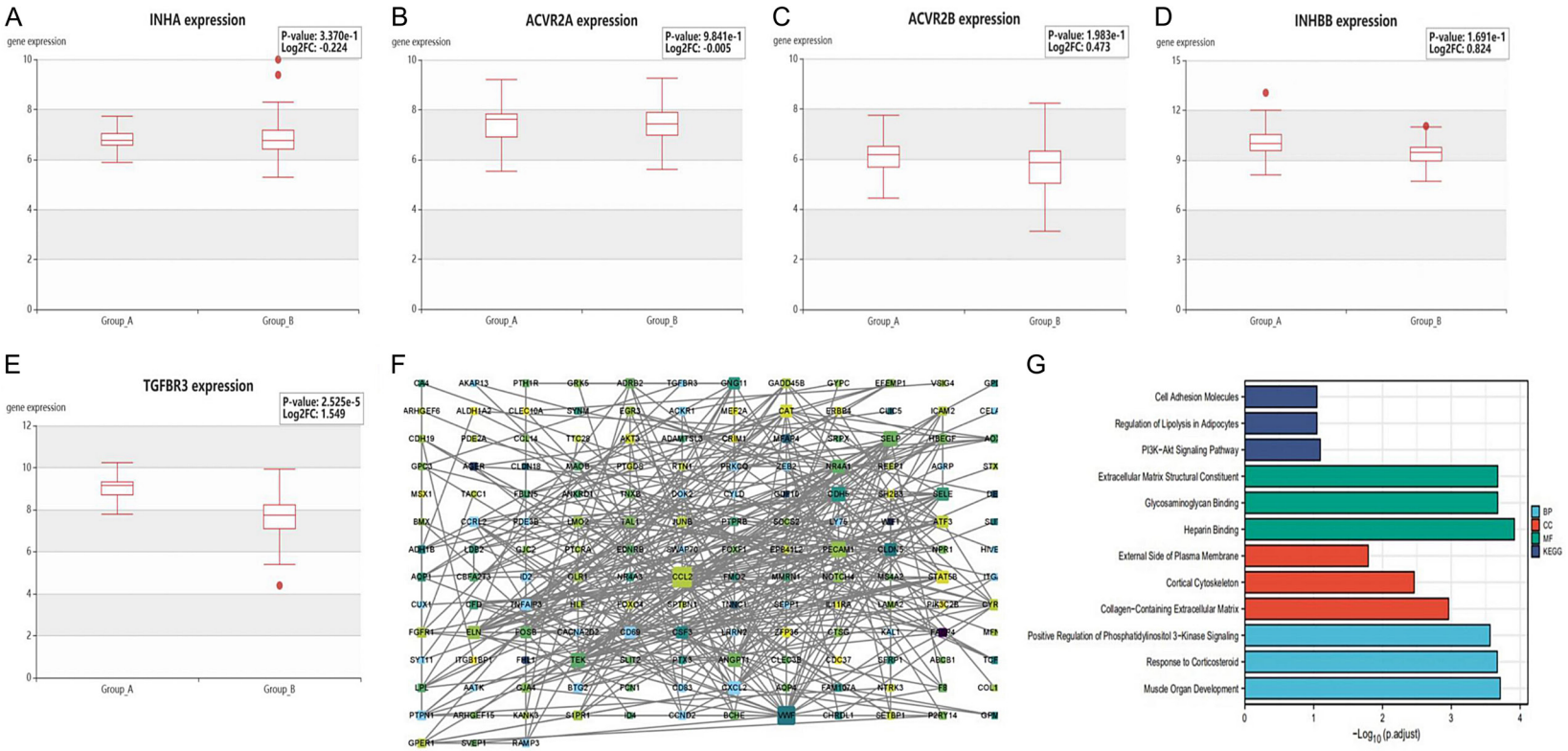

Figure 5. The differential expression and co-expression of Inhibin B in metastasis samples of LUAD. A-E. The differential expression of INHB in metastasis samples of LUAD (HCMDB). F. The genes co-expressed with TGFBR3 from HCMDB constructed in the STRING database. G. GO/KEGG pathway enrichment analysis of genes co-expressed with TGFBR3.

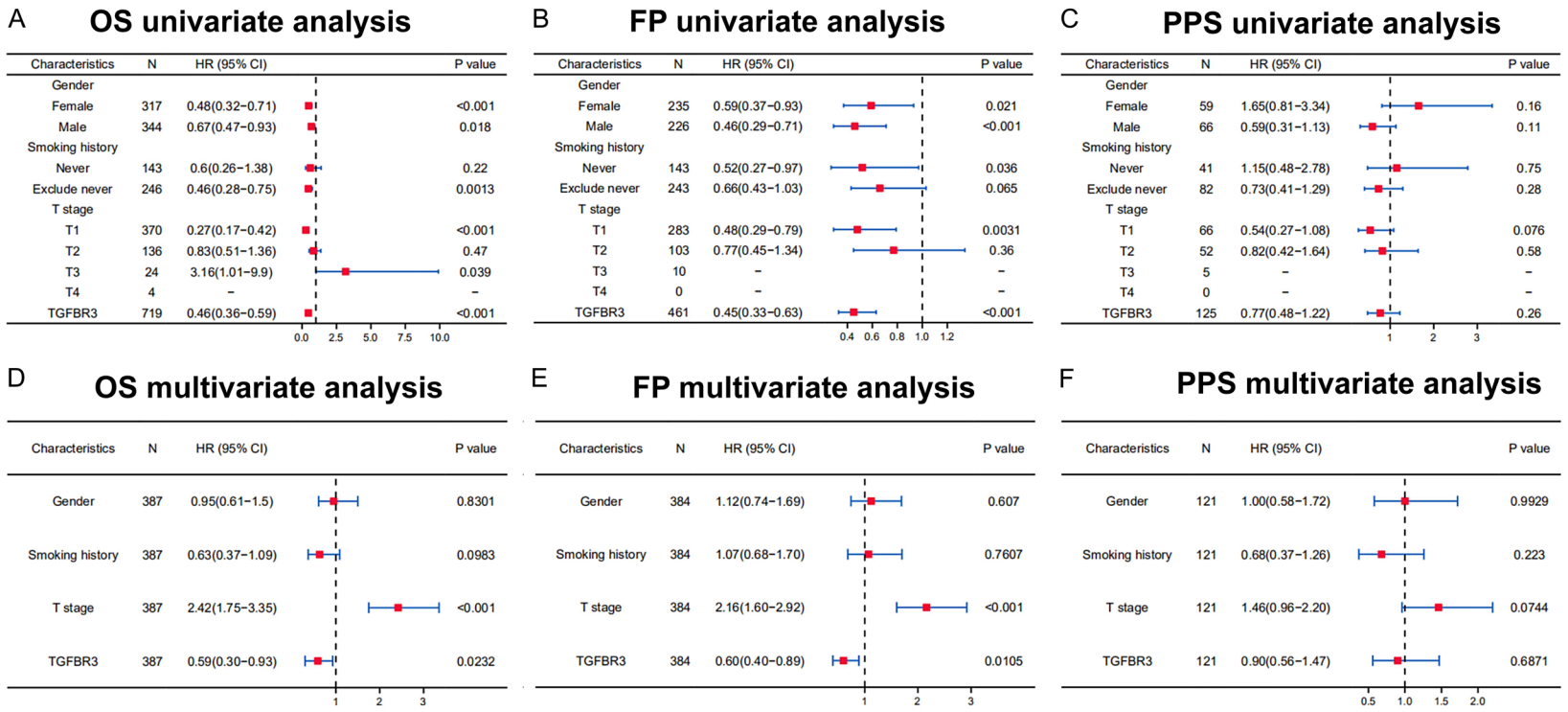

Figure 6. Univariate and multivariate regression analysis of TGFBR3 and clinicopathologic parameters with OS, FP and PPS in LUAD patients. Red squares represent hazard ratio. Short bars appear due to limited sample size for parameters and hazard ratio cannot be calculated. OS: overall survival; FP: first progression; PPS: post-progressive survival.

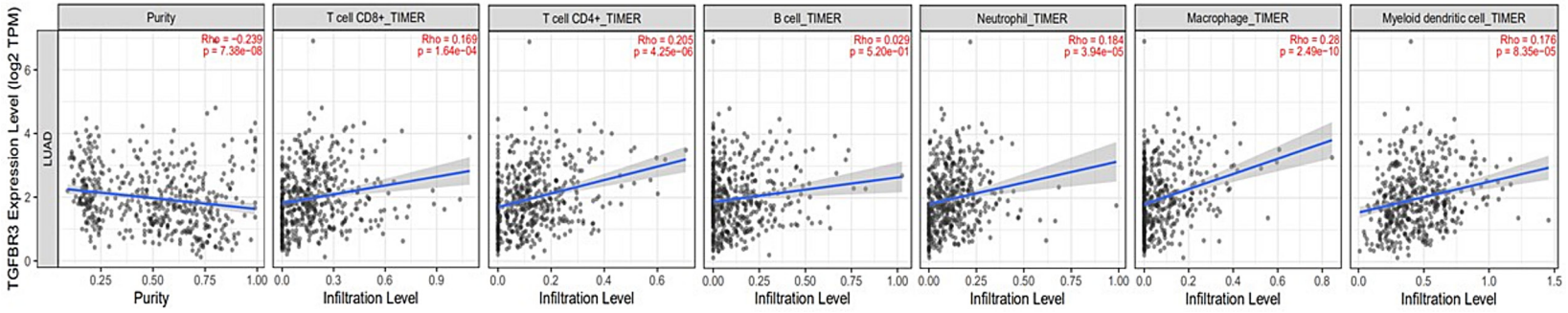

Figure 7. Correlation of TGFBR3 expression with immune infiltration level in LUAD. TGFBR3 expression has significant negative correlation with tumor purity and significant positive correlation with infiltrating levels of CD8+ T cell, CD4+ T cell, neutrophil, macrophage and myeloid dendritic cell.

Table 4. Correlations between TGFBR3 and Gene Markers of Immune Cells in TIMER

Cell type	Gene marker	None		Purity	
		Rho	*P*	*Rho*	*P*
B cell	CD19	0.205	***	0.103	0.023
	CD20 (KRT20)	0.140	*	0.124	0.006
	CD38	0.132	*	0.053	0.239
CD8+ T cell	CD8A	0.105	0.017	0.002	0.958
	CD8B	0.054	0.217	-0.036	0.422
Tfh	CXCR5	0.303	***	0.215	***
	BCL6	0.225	***	0.221	***
	ICOS	0.214	***	0.102	0.024
Th1	IL12RB2	0.011	0.811	-0.044	0.325
	T-bet (TBX21)	0.207	***	0.121	**
	STAT6	0.266	***	0.287	***
	TNF-α	0.165	**	0.068	0.129
	WSX1 (IL27RA)	0.15	**	0.102	0.024
Th2	CCR3	0.103	0.020	0.059	0.19
	STAT6	0.266	***	0.287	***
	GATA3	0.328	***	0.268	***
Th9	TGFBR2	0.603	***	0.585	***
	IRF4	0.255	***	0.165	**
	PU.1 (SPI1)	0.275	***	0.207	***
Th17	STAT3	0.28	***	0.292	***
	IL21R	0.201	***	0.09	0.046
	IL23R	0.281	***	0.238	***
	IL17A	-0.020	0.643	-0.085	0.059
Th22	CCR10	-0.007	0.877	-0.021	0.645
	AHR	0.312	***	0.286	***
Treg	FOXP3	0.201	***	0.105	0.020
	CD25 (IL2RA)	0.16	**	0.076	0.094
	CCR8	0.255	***	0.172	**
T cell exhaustion	PD1 (PDCD1)	0.054	0.223	-0.061	0.176
	CTLA4	0.131	*	0.018	0.682
	LAG3	0.023	0.601	-0.075	0.096
	TIM3 (HAVCR2)	0.215	***	0.134	**
Macrophage	CD68	0.296	***	0.249	***
	CD11b (ITGAM)	0.345	***	0.294	***
M1	INOS (NOS2)	0.25	***	0.217	***
	COX2 (PTGS2)	0.139	*	0.144	**
M2	CD163	0.324	***	0.274	***
	ARG1	0.214	***	0.215	***
	MRC1	0.439	***	0.407	***
TAM	CCL2	0.245	***	0.18	***
	CD80	0.290	***	0.213	***
	CD86	0.259	***	0.185	***
Monocyte	CD14	0.158	**	0.087	0.053
	CD16 (FCGR3B)	0.226	***	0.186	***
Neutrophil	CD66b (CEACAM8)	0.348	***	0.359	***

expression was down-regulated in LUAD tissue (21 low expression cases of 41 cases, 51.2%) compared with non-cancerous epithelium (8 of 41, 19.5%), with statistical significance (χ^2=9.016, *P*=0.003). Furthermore, TGFBR3 expression negatively correlates with lymph node metastases and clinical stage in patients with LUAD (*P*=0.019, *P*=0.012, respectively), which validated the above database analysis that low TGFBR3 was associated with LUAD metastasis (**Table 5**).

Low expression of TGFBR3 may promote the metastasis of LUAD

After a thorough analysis of the above data, we found that low TGFBR3 expression in LUAD can affect the tumor microenvironment through PI3K-Akt signaling, regulation of adipocyte lipolysis and cell adhesion molecules. The microenvironment, which regulates adipocyte lipolysis and cell adhesion molecules, leads to a diminish in macrophages and dendritic cells. This leads to a reduction of macrophages and dendritic cells, resulting in immune escape or immunodeficiency, which promotes distant metastasis of lung adenocarcinoma (**Figure 9**).

Discussion

Inhibins have a similar β subunit as activins and usually perform biological functions by inhibiting activins. INHB is produced principally by granulosa cells of growing ovarian follicles and testicular sertoli cells, so related research is mainly on reproductive system diseases [23]. ACVR2A/B

	CD15 (FUT4)	0.291	***	0.256	***
	MPO	0.257	***	0.213	***
NK	CD7	0.008	0.856	-0.088	0.051
	XCL1	-0.022	0.616	-0.075	0.095
	KIR3DL1	0.068	0.123	0.022	0.63
DC	CD1C	0.421	***	0.383	***
	CD141 (THBD)	0.46	***	0.442	***

Note: Tfh, follicular helper T cell; Th, T helper cell; Treg, regulatory T cell; TAM, tumor-associated-macrophage; NK, natural killer cell; DC, dendritic cell; None, correlation without adjustment; Purity, correlation adjusted for tumor purity; *Rho*, R value of Spearman's correlation. *P<0.01, **P<0.001, ***P<0.0001.

is the receptor of the βB subunit of INHB, and TGFBR3 may be the co-receptor of the α subunit of INHB [24]. We have found that INHB was differentially expressed in LUAD tissues (data not shown). Therefore, we performed bioinformatics to analyze whether INHB and its receptors would influence the progression of LUAD.

We analyzed the role of INHB and its receptor expression during tumorigenesis and progression, as well as metastasis and prognosis of LUAD, based on several databases, including TCGA, HCMDB, and TIMER. Not surprisingly, INHB and its receptors were differentially expressed in LUAD, where TGFBR3 was downregulated. In addition, INHB and its receptors could predict the tumorigenesis of LUAD, and TGFBR3 had preferable specificity and sensitivity (**Figure 1**; **Table 1**). Studies showed that Ovarian and testicular malignant germ cell tumors expressed positive staining for inhibin/activing α, βA and βB subunits [25]. INHBB was strongly enhanced in some hepatocellular carcinoma [26]. Pancreatic cancer tissues markedly over-expressed the inhibin βA subunit, ACVR1 and ACVR2, whereas the βB subunit was only moderately increased compared to standard pancreatic samples [27]. INHBA mRNA and protein expression were commonly elevated in primary human NSCLC. They were a critical autocrine factor maintaining mesenchymal properties of cancer-initiating cells to promote metastasis in NSCLC [28]. Eduardo Listik et al. found using bioinformatic analysis lung adenocarcinoma and renal clear cell carcinoma with high INHA expression [29]. All of the above indicated that INHB and its receptors were differentially expressed in tumor tissues.

INHB and its receptors were associated with tumor progression and prognosis. The increased INHA expression was a separate adverse prognostic factor in ovarian clear cell carcinoma. Loss of ACVR2A plays an essential role in cancer progression and distant metastasis and may be a prognostic marker for patients with colon cancer [30, 31]. Comparison of mRNA from benign neuroblastic tumors and neuroblastomas revealed that expression of TGFBR3 decreased with advancing stage of neuroblastoma and this loss correlated with a poorer prognosis [32]. Our analysis also showed that INHA, ACVR2A, and TGFBR3 were significantly correlated with pathological stage from the GEPIA database and had diagnostic values (**Table 3**), and these were significantly correlated with lymphatic metastasis from the UALCAN database (**Figure 2**). Furthermore, the expression of INHA, ACVR2A and TGFBR3 were significantly associated with the prognosis of OS and FP in LUAD patients (**Figure 3**). Listik et al. also found that using bioinformatic analysis INHA and TGFBR3 were predictors of survival in lung cancers [29]. In the multivariate Cox model, TGFBR3 expression was an independent factor correlated with OS and FP (**Figure 6**).

Furthermore, we confirmed that TGFBR3 and ACVR2A expression is associated with LUAD metastasis in a single-cell sequencing database (**Figure 4**). Orthotopic inoculation experiments using immunocompromised mice indicated that low TGFBR3 expression in clear-cell renal cell carcinoma (ccRCC) cells enhanced primary tumor formation and lung metastasis, loss of TGFBR3 endows ccRCC cells with multiple metastatic abilities through TGF-β-dependent and independent pathways [33]. These results suggested that the low expression of TGFBR3 was related to the metastasis of the tumor. We further validated the results with a tumor metastasis database, HCMDB. Only TGFBR3 had differential expression in LUAD metastasis, TGFBR3 may influence functions of extracellular matrix structural constituent, glycosaminoglycan binding and heparin-binding, through PI3K-Akt signaling, regulation of lipolysis in adipocytes and cell adhesion molecules pathways, thereby affected the metastasis of LUAD (**Figure 5**).

GPC3 and FGFR1 were also identified as the critical proteins that interact with TGFBR3 molecules in LUAD metastasis based on analysis

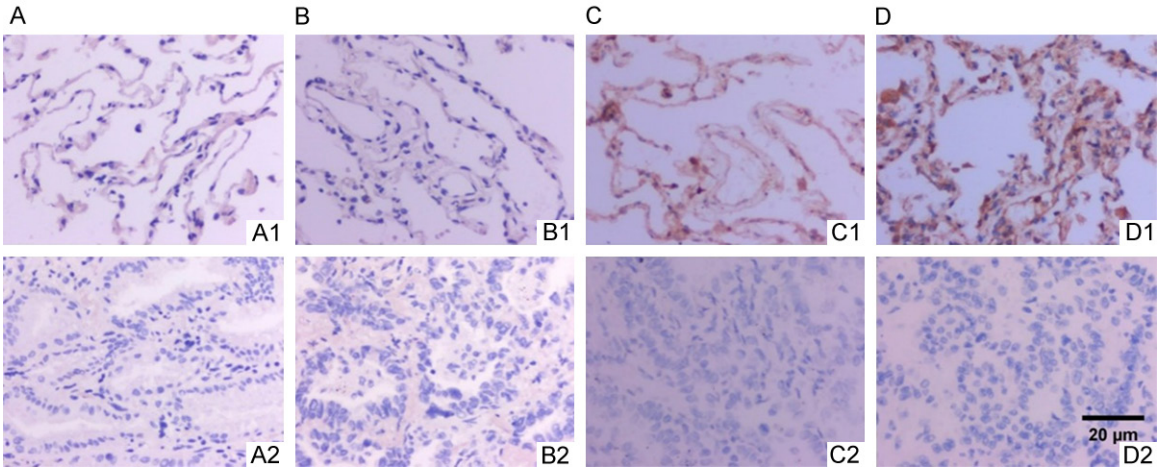

Figure 8. The expression of TGFBR3 in part of LUAD and non-cancerous tissues was detected by immunohistochemistry (×200). (A-D) is immunohistochemical images of corresponding cancer and non-cancerous tissues in 4 patients with LUAD. (A1, B1) TGFBR3 was low expression in non-cancerous tissues; (C1, D1) TGFBR3 was high expression in non-cancerous tissues; (A2, B2, C2, D2) TGFBR3 was low expression in LUAD.

Table 5. Correlation between TGFBR3 and clinicopathologic parameters in patients with LUAD

Characteristics	Sample (n)	TGFBR3 expression		χ^2	*P* value
		upregulate	downregulate		
Tissue					
cancer tissues	41	20	21	9.016	0.003
non-cancerous tissues	41	33	8		
Gender					
Male	24	11	13	0.201	0.654
Female	17	9	8		
Age					
≤60	15	7	8	0.042	0.837
>60	26	13	13		
Lymphatic metastasis					
Metastasis	20	6	14	5.512	0.019
No metastasis	21	14	7		
Stage					
Stages I+II	27	17	10	6.366	0.012
Stages III+IV	14	3	11		
T stage					
T1+T2	34	16	18	0.005	0.943
T3+T4	7	4	3		

with STING software. Glypican-3 (GPC3), a proteoglycan bound to the cell membrane by a GPI anchor, is involved in the control of proliferation and survival. GPC3 could return mesenchymal-like breast cancer cells to an epithelial phenotype, impair in vivo metastasis, and induces tumor dormancy through p38 MAPK signaling activation [34]. FGFR1 promoted the processes of EMT through AKT/MAPK signaling by targeting CCND1 and prompted cell proliferation and metastasis in lung squamous cell cancer [35]. Combined with the above results, TGFBR3 may regulate the metastasis of LUAD through the PI3K-Akt pathway (**Figure 9**).

Immunotherapy has been used clinically against tumor metastasis, and studies have shown that metastasis could be prevented early by increasing the level of immune cells in primary tumors and circulation. However, due to immune escape from tumor cells, T cells are often insufficient for complete control of the metastatic disease [36, 37]. Therefore, overcoming immunosuppression may be an essential measure to improve anti-metastatic immunotherapy.

Recently, studies have shown that the tumor immune microenvironment plays a critical role in tumorigenesis and metastasis. Tumor infiltrating immune cells including tumor biology's primary regulators in the tumor microenvironment. The results shown that TGFBR3 expres-

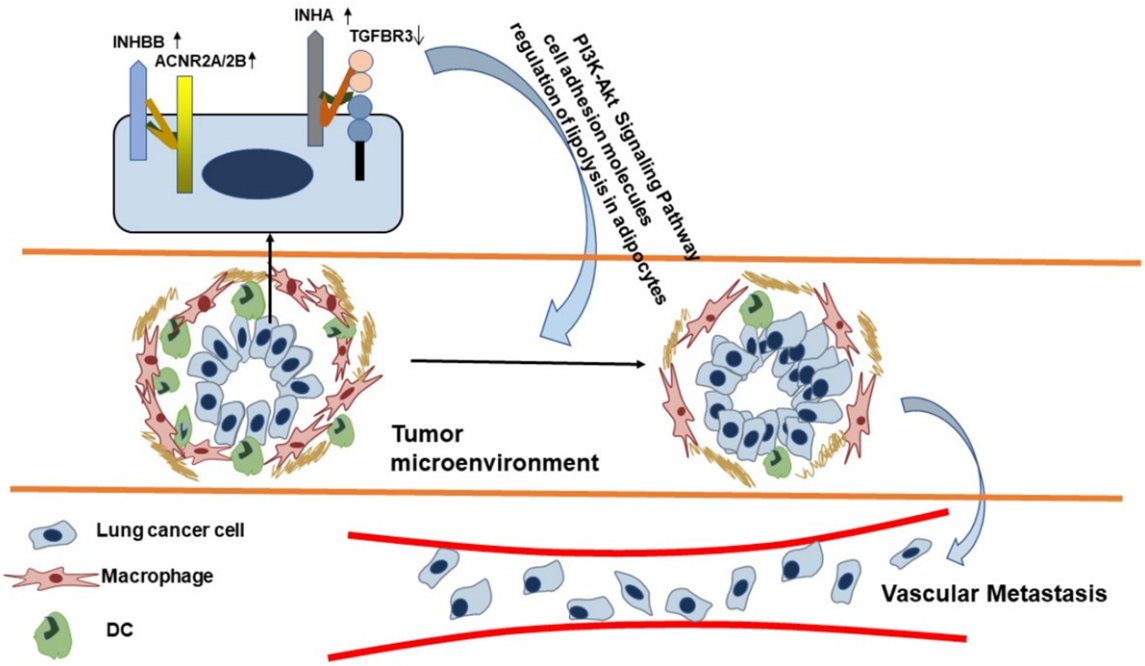

Figure 9. Low expression of TGFBR3 may promote the metastasis of LUAD. The low expression of TGFBR3 in LUAD may impact the tumor microenvironment through the PI3K-Akt signaling, regulation of lipolysis in adipocytes and cell adhesion molecules, leading to the reduction of macrophages and dendritic cells, and further promoting the distant metastasis of lung adenocarcinoma cells.

sion positively correlated with CD8+ T cells, CD4+ T cells, neutrophils, macrophages, and dendritic cells in LUAD. The TGFBR3 expression level was significantly correlated with Th, Macrophage, TAM, DC, and Neutrophil cells markers. Further analysis of infiltrating immune cell markers revealed that M2 macrophage markers, such as MRC1, and DC markers, such as CD141, which are important antigen-presenting cells, were moderately correlated with TGFBR3 expression. In contrast, Th9 genetic markers, such as TGFBR2, were strongly correlated with TGFBR3 levels, suggesting that the tumor immune microenvironment in LUAD is regulated by TGFBR3 (**Figure 7** and **Table 4**), which may ultimately promote distant metastasis in LUAD and affect patient survival (**Figure 9**). In the early stages of progression of many human cancers, TGFBR3 expression was downregulated [38], and deletion of tumor-expressed TGFBR3 increases TGF-β signaling within the local DC population, and alterations in these DC populations mediate Treg infiltration and suppression of antitumor immunity, creating a state of immune tolerance that further promotes tumor progression and metastasis [39]. Finally, we validated that TGFBR3 expression was down-regulated by examining tissues from 41 LUAD patients and that TGFBR3 expression negatively related to lymphatic metastasis and clinical staging (**Figure 8** and **Table 5**).

This study has improved the comprehension of the relationship between TGFBR3 and lung adenocarcinoma, but still has some limitations. Most of these analytical data were obtained from the platform database and only preliminary in vivo validation has been performed. The mechanism and role of TGFBR3 in tumor growth, metastasis and immune infiltration in vitro needs further investigation.

Conclusions

In conclusion, the expression of TGFBR3 was down-regulated, low TGFBR3 expression was related to poor prognosis and tumor metastasis, and correlated with decreased immune cells infiltration in LUAD. Our results suggested that TGFBR3 could serve as a potential novel prognostic biomarker for LUAD. The low expression of TGFBR3 in LUAD may influence the tumor microenvironment the PI3K-Akt signaling pathway, leading to the reduction of macrophages and dendritic cells, resulting in immune escape or immune deficiency, thus promoting

the distant metastasis of LUAD cells and influencing patient survival. These findings will have potential value not only for the role of TGFBR3 but also for its translational application in LUAD prognosis and immunotherapy.

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Disclosure of conflict of interest

None.

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References

[1] Li F, He H, Qiu B, Ji Y, Sun K, Xue Q, Guo W, Wang D, Zhao J, Mao Y, Mu J and Gao S. Clinicopathological characteristics and prognosis of lung cancer in young patients aged 30 years and younger. J Thorac Dis 2019; 11: 4282-4291.

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[3] Aggarwal A, Lewison G, Idir S, Peters M, Aldige C, Boerckel W, Boyle P, Trimble E, Roe P, Sethi T, Fox J and Sullivan R. The state of lung cancer research: a global analysis. J Thorac Oncol 2016; 11: 1040-1050.

[4] Ma J, Ward EM, Smith R and Jemal A. Annual number of lung cancer deaths potentially avertable by screening in the United States. Cancer 2013; 119: 1381-5.

[5] Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, Cho J, Suh J, Capelletti M, Sivachenko A, Sougnez C, Auclair D, Lawrence MS, Stojanov P, Cibulskis K, Choi K, de Waal L, Sharifnia T, Brooks A, Greulich H, Banerji S, Zander T, Seidel D, Leenders F, Ansén S, Ludwig C, Engel-Riedel W, Stoelben E, Wolf J, Goparju C, Thompson K, Winckler W, Kwiatkowski D, Johnson BE, Jänne PA, Miller VA, Pao W, Travis WD, Pass HI, Gabriel SB, Lander ES, Thomas RK, Garraway LA, Getz G and Meyerson M. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 2012; 150: 1107-1120.

[6] Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ Jr, Wu YL and Paz-Ares L. Lung cancer: current therapies and new targeted treatments. Lancet 2017; 389: 299-311.

[7] Schild SE, Tan AD, Wampfler JA, Ross HJ, Yang P and Sloan JA. A new scoring system for predicting survival in patients with non-small cell lung cancer. Cancer Med 2015; 4: 1334-1343.

[8] Pangas SA and Woodruff TK. Activin signal transduction pathways. Trends Endocrinol Metab 2000; 11: 309-314.

[9] Mathews LS and Vale WW. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 1991; 65: 973-982.

[10] Lebrun JJ and Vale WW. Activin and inhibin have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human erythroid differentiation. Mol Cell Biol 1997; 17: 1682-1691.

[11] Martens JW, de Winter JP, Timmerman MA, McLuskey A, van Schaik RH, Themmen AP and de Jong FH. Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells. Endocrinology 1997; 138: 2928-2936.

[12] Chapman SC and Woodruff TK. Betaglycan localization in the female rat pituitary: implications for the regulation of follicle-stimulating hormone by inhibin. Endocrinology 2003; 144: 5640-5649.

[13] Zou G, Ren B, Liu Y, Fu Y, Chen P, Li X, Luo S, He J, Gao G, Zeng Z, Xiong W, Li G, Huang Y, Xu K and Zhang W. Inhibin B suppresses anoikis resistance and migration through TGF-β signaling pathway in nasopharyngeal carcinoma. Cancer Sci 2018; 109: 3416-3427.

[14] Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pander A and Chinnaiyan AM. Oncomine: a cancer microarray database and integrated data-mining platform. Neoplasia 2004; 6: 1-6.

[15] Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK and Varambally S. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia 2017; 19: 649-658.

[16] Tang Z, Li C, Kang B, Gao G, Li C and Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017; 45: W98-W102.

[17] Győrffy B, Surowiak P, Budczies J and Lánczky A. Online survival analysis software to assess

the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS One 2013; 8: e82241.
[18] Yu G, Wang LG, Han Y and He QY. ClusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 2012; 16: 284-287.
[19] Yuan H, Yan M, Zhang G, Liu W, Deng C, Liao G, Xu L, Luo T, Yan H, Long Z, Shi A, Zhao T, Xiao Y and Li X. CancerSEA: a cancer single-cell state atlas. Nucleic Acids Res 2019; 47: D900-D908.
[20] Zheng G, Ma Y, Zou Y, Yin A, Li W and Dong D. HCMDB: the human cancer metastasis database. Nucleic Acids Res 2018; 46: D950-D955.
[21] Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ and Mering CV. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 2019; 47: D607-D613.
[22] Doncheva NT, Morris JH, Gorodkin J and Jensen LJ. Cytoscape stringApp: network analysis and visualization ofproteomics data. J Proteome Res 2019; 18: 623-632.
[23] Anawalt BD, Bebb RA, Matsumoto AM, Groome NP, Illingworth PJ, Mcneilly AS and Bremner WJ. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. J Clin Endocrinol Metab 1996; 81: 3341-3345.
[24] Bernard DJ, Smith CL and Brlé E. A tale of two proteins: betaglycan, IGSF1, and the continuing search for the inhibin B receptor. Trends Endocrinol Metab 2020; 31: 37-45.
[25] Cobellis L, Cataldi P, Reis FM, Palo GD and Petraglia F. Gonadal malignant germ cell tumors express immunoreactive inhibin/activin subunits. Eur J Endocrinol 2001; 145: 779-784.
[26] Frost K, Seir K, Lackner A, Grusch M, Graslkraupp B, Schulte-Hermann R and Rodgarkia-Dara C. Inhibin/activin expression in human and rodent liver: subunits α and βB as new players in human hepatocellular carcinoma? Br J Cancer 2011; 104: 1303-1312.
[27] Kleeff J, Ishiwata T, Friess H, Büchler M and Korc M. Concomitant over-expression of activin/inhibin beta subunits and their receptors in human pancreatic cancer. Int J Cancer 1998; 77: 860-868.
[28] Wamsley JJ, Kumar M, Allison DF, Clift SH, Holzknecht CM, Szymura SJ, Hoang SA, Xu X, Moskaluk CA, Jones DR, Bekiranov S and Mayo MW. Activin upregulation by NF-κB is required to maintain mesenchymal features of cancer stem-like cells in non-small cell lung cancer. Cancer Res 2015; 75: 426-435.
[29] Listik E, Horst B, Choi AS, Lee NY and Mythreye K. A bioinformatic analysis of the inhibin-betaglycan-endoglin/CD105 network reveals prognostic value in multiple solid tumors. PLoS One 2021; 16: e0249558.
[30] Kusumoto S, Kurashige M, Ohshima K, Tahara S, Matsui T, Nojima S, Hattori S and Morii E. An immature inhibin-α-expressing subpopulation of ovarian clear cell carcinoma cells is related to an unfavorable prognosis. Cancer Med 2021; 10: 1485-1500.
[31] Zhuo C, Hu D, Li J, Yu H, Lin X, Chen Y, Zhuang Y, Li Q, Zheng X and Yang C. Downregulation of activin a receptor type 2A is associated with metastatic potential and poor prognosis of colon cancer. J Cancer 2018; 9: 3626-3623.
[32] Knelson EH, Gaviglio AL, Tewari AK, Armstrong MB, Mythreye K and Blobe GC. Type III TGF-β receptor promotes FGF2-mediated neuronal differentiation in neuroblastoma. J Clin Invest 2013; 123: 4786-4798.
[33] Nishida J, Miyazono K and Ehata S. Decreased TGFBR3/betaglycan expression enhances the metastatic abilities of renal cell carcinoma cells through TGF-β-dependent and -independent mechanisms. Oncogene 2018; 37: 2197-2212.
[34] Guereño M, Delgado Pastore M, Lugones AC, Cercato M, Todaro L, Urtreger A and Peters MG. Glypican-3 (GPC3) inhibits metastasis development promoting dormancy in breast cancer cells by p38 MAPK pathway activation. Eur J Cell Biol 2020; 99: 151096.
[35] Yang Y, Lu T, Li Z and Lu S. FGFR1 regulates proliferation and metastasis by targeting CCND1 in FGFR1 amplified lung cancer. Cell Adh Migr 2020; 14: 82-95.
[36] Nicol AJ, Tokuyama H, Mattarollo SR, Hagi T, Suzuki K, Yokokawa K and Nieda M. Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. Br J Cancer 2011; 105: 778-786.
[37] Talmadge JE. Immune cell infiltration of primary and metastatic lesions: mechanisms and clinical impact. Semin Cancer Biol 2011; 21: 131-138.
[38] Finger EC, Turley RS, Dong M, How T, Fields TA and Blobe GC. TβRIII suppresses non-small cell lung cancer invasiveness and tumorigenicity. Carcinogenesis 2008; 29: 528-535.
[39] Hanks BA, Holtzhausen A, Evans KS, Jamieson R, Gimpel P, Campbell OM, Hector-Greene M, Sun L, Tewari A, George A, Starr M, Nixon AB, Augustine C, Beasley G, Tyler DS, Osada T, Morse MA, Ling L, Lyerly HK and Blobe GC. Type III TGF-β receptor downregulation generates an immunotolerant tumor microenvironment. J Clin Invest 2013; 123: 3925-3940.