GRAP2 is a Prognostic Biomarker and Correlated with Immune Infiltration in Lung Adenocarcinoma

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Abstract

Background: GRAP2 is an adaptor protein involved in leukocyte signal activation; however, the prognostic value of GRAP2 and its correlation with immune cell infiltration in lung adenocarcinoma (LUAD) is unclear.

Methods: Original data were downloaded from the TCGA database and Gene Expression Omnibus (GEO) database. GRAP2 expression was analyzed with the TCGA and TIMER databases. We evaluated the influence of GRAP2 on clinical prognosis using the Kaplan-Meier plotter, GEO database and GEPIA database. TIMER and TISIDB databases were used to investigate correlations between GRAP2 expression and cancer immune characteristics. Finally, we confirmed the expression of GRAP2 in LUAD by immunohistochemistry staining.

Results: Transcription levels of GRAP2 were significantly lower in several human cancers, including LUAD, than in adjacent normal tissues. We also found that tumor tissues have lower protein expression levels of GRAP2 compared with adjacent normal tissues in LUAD by immunohistochemistry staining. The down-regulated GRAP2 was associated with poorer overall survival, pathologic stage, T stage, N stage and primary therapy outcome in LUAD. Mechanically, we found a hub gene set that included a total of 91 genes co-expressed with GRAP2, which were closely related to immune response in LUAD. Expression levels of GRAP2 were positively correlated with infiltrating levels of B cells, $CD8^+$ T cells, dendritic cells, eosinophils, macrophages, mast cells, Th2 cells, Th1 cells, Th17 cells, NK cells and neutrophils. GRAP2 expression levels also affected the cumulative survival time of B cells and dendritic cells. GRAP2 expression is positively correlated with multiple immune markers, chemokines, chemokine receptors and MHC molecules of LUAD.

Conclusions: These findings suggest that GRAP2 is a tumor suppressor gene and can be used as a prognostic biomarker for assessing prognosis and immune infiltration level in LUAD.

1. Introduction

Lung cancer is one of the malignant tumors with the highest incidence and worse prognosis in the world [1]. Lung adenocarcinoma (LUAD) is a common subtype of lung cancer [2–4]. Despite improvements in systemic treatments for patients, the 5-year survival rate of LUAD patients is 15% [5]. Therefore, it is crucial to explore useful prognostic biomarkers and therapeutic targets to against LUAD in the diagnosis, prevention and treatment.

The tumor microenvironment (TME) contains extracellular matrix, stromal cells and tumor infiltrating immune cells (TIICs) that shape cancer development [6]. Increased evidences indicate that TIICs determine the success of immunotherapy and affects the prognosis of patients [7]. It is currently unclear which factors drive immune infiltration in LUAD. Therefore, it is necessary to find new biomarkers to assess immune infiltration in LUAD.

GRAP2(Gads/Mona/GrpL/Grf40) is an important adaptor protein in protein-tyrosine kinase signal transduction of leukocytes [8]. Studies show that GRAP2 is highly expressed in lymphoid organs and T lymphocytes. GRAP2 forms signal complexes with different signal molecules to mediate the activation and signal transduction of T cells [9]. At present, research of GRAP2 mainly focuses on the immune system[10, 11]. The role of GRAP2 in tumors and its relationship with immune infiltration are largely unknown. There is only one study on the role of GRAP2 in cancer, research indicate that GRAP2 directly interacts with the tyrosine kinase RET and inhibits RET-induced NF-κB activation in a dose-dependent manner in medullary thyroid cancer cells [12].

In recent years, more and more platforms and open databases have enabled tumor researchers to use multiple data for cancer bioinformatics analysis[4, 13]. In order to better explore the role of GRAP2 in LUAD, we assessed the relationship between GRAP2 expression and the prognosis of LUAD by TCGA, TIMER, and Kaplan-Meier plotter database. In addition, we also investigated the correlation between GRAP2 expression and immune infiltration by the TIMER and TISIDB databases. Our findings revealed that GRAP2 can be used as a biomarker for assessing prognosis and immune infiltration levels in LUAD.

2. Materials And Methods

Patient data set

FPKM data (535 LUAD samples, 59 adjacent normal samples) and relevant clinical data were downloaded from the TCGA database (https://cancergenome.nih.gov) [14]. Dataset GSE37745 (196 LUAD samples) downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.n1m.nih.gov/gds/?term=).

Survival analysis

The survival rates and the hazard ratio (HR) analysis were performed by Kaplan-Meier plotter database (http://kmplot.com/analysis/) [15] and Gene Expression Profiling Interactive Analysis (GEPIA) database (http:GEPIA cancer-pku.cn) [16].

Correlation heat map and protein-protein interaction (PPI) network analysis

The GeneMANIA database (http://www.genemania.org) [17] was used to generate the correlation heat map for the co-expressed genes with GRAP2. The STRING database (https://string-db.org) was used to obtain PPI data based on protein interaction and signal pathways, Cytoscape 3.7.2 was used to construct the network.

GEPIA database analysis

GEPIA (http://gepia.cancer-pku.cn/index.html) [16] was used to screen genes that are positive or negative correlated with GRAP2 by “Similar Genes” module. The “Survival” module in GEPIA was used to explore genes that associated with LUAD prognosis.

Linked omics database analysis

The Linked Omics database (http://www.linkedomics.org) [18] contains 32 tumor types from the TCGA database and a total of 11158 patients with multi-omics data and clinical data. In this study, the gene ontology biological process (GO_BP) and KEGG pathway were performed by the Linked Omics database.

Timer database analysis

In this study, the TIMER database (http://www.cistrome.shinyapps.io) [19] was used to examine the gene expression in normal tissues and tumor tissues in a variety of tumors. The abundance of TIIC from the gene expression profile of LUAD samples in the TCGA data also analyzed by this database.

TISIDB database analysis

The TISIDB database (http://cis.hku.hk/TISIDB) [20] is a highly cited portal allows researchers to interactively explore tumor and immune system interaction. In this study, the "Immunomodulator" module of TISIDB database was utilized to explore the correlation between GRAP2 expression and MHC molecules. In order to investigate the relationship between GRAP2 and chemokine/chemokine receptor expression, we examined the chemokine/chemokine receptor expression level of TIIC by the "chemokine" module.

Western blot

LUAD cells were lysed using RIPA lysis buffer (Medchemexpress, China). The protein lysate was separated by SDS-PAGE gel (Invitrogen) and printed on PVDF membrane (Millipore, USA) for analysis [21]. Anti-GRAP2 (1:2000 dilution, Cambridge, MA, USA, ab224613) and anti-GAPDH (1:5000 dilution, Proteintech, China, 60004-1-Ig) were incubated at 4°C for overnight. HRP-labeled secondary antibody (1:2000 dilution, Beyotime, China, A0181) was kept at room temperature for 2 hours. Western blot analysis results were performed with Image J software.

Immunohistochemistry (IHC) staining

Seventy-six paraffin-embedded LUAD tissues and para-carcinoma tissues were performed for IHC staining. Tissues were incubated with anti-GRAP2 antibody (1:2000 dilution, MA, USA, ab224613) overnight at 4°C. Phosphate-buffered saline (PBS) was used to wash three times, tissues were then incubated with HRP-labeled goat anti-mouse secondary antibody (1:2000 dilution, Beyotime, China, A0181) for 0.5 h at room temperature. Stained IHC sections were counterstained with hematoxylin (Beyotime). Slides were scanned using a Microscope (FisherScientific) and ImageJ software was utilized to analyze the intensity of staining [22].

Statistical analysis

The original data were calculated using GraphPad Prism (version 8.0). The correlation of gene expression was evaluated by Spearman’s correlation and statistical significance. Use univariate and multivariate Cox

analysis to screen for potential prognostic factors. The MannWhitney test and an independent t test were used to analyze the difference between two groups of data. the difference among different groups were calculated using one-way ANOVA with the post hoc Tukey test and chi-square test. *, * *, and * * * indicate P <0.05, P <0.01, and P <0.001, respectively.

3. Results

3.1 Decreased GRAP2 expression in LUAD

The TIMER database was used to assess the transcriptional level of GRAP2 in different tumors and normal tissues. Results showed that GRAP2 transcriptional level was significantly lower in tumor tissues in matched normal tissues, such as bladder urothelial carcinoma, breast invasive carcinoma, colon adenocarcinoma, LUAD, lung squamous cell carcinoma (LUSC), prostate adenocarcinoma, rectum adenocarcinoma. However, higher transcriptional level was observed in tumors such as esophageal carcinoma and kidney renal clear cell carcinoma (Figure 1A).

We further used the TCGA database to examine the transcriptional level of GRAP2 in tumor tissues compared with adjacent normal tissues in LUAD. The results showed that the transcriptional level of GRAP2 in tumor tissues was significantly lower than that in unmatched adjacent normal tissues (P<0.01) (Figure 1B). These results were verified in matched tumor tissues and adjacent normal tissues (Figure 1C). The similar results were found in LUSC (Supplementary Figure 1A and 1B).

Table 1 summarized the correlation between GRAP2 expression and clinical characteristics in LUAD. The results showed that the low expression of GRAP2 is associated with late T stage, N stage, pathologic stage, and worse primary therapy outcome (Figure 1D-1G and Table 1). The protein expression level of GRAP2 was further investigated by immunohistochemical staining, we found that the GRAP2 protein level was obviously decreased in LUAD tissues compared with adjacent normal tissues (Figures 2A and 2B). Finally, we investigated GRAP2 expression in LUAD cell lines, data showed that GRAP2 mRNA level in three LUAD cell lines (A549, H1975 and H1299) was significantly down-regulated compared with that in a normal lung epithelial cell line (BEAS -2B) (Figure 2C).

Table 1
Correlation between GRAP2 expression and the clinicopathological features of the LUAD cases from TCGA.

Characteristic	Low expression of GRAP2	High expression of GRAP2	p
n	267	268	
T stage, n (%)			< 0.001
T1	65 (12.2%)	110 (20.7%)	
T2	160 (30.1%)	129 (24.2%)	
T3	28 (5.3%)	21 (3.9%)	
T4	14 (2.6%)	5 (0.9%)	
N stage, n (%)			0.002
N0	158 (30.4%)	190 (36.6%)	
N1	53 (10.2%)	42 (8.1%)	
N2	50 (9.6%)	24 (4.6%)	
N3	1 (0.2%)	1 (0.2%)	
M stage, n (%)			0.330
M0	187 (48.4%)	174 (45.1%)	
M1	16 (4.1%)	9 (2.3%)	
Pathologic stage, n (%)			0.002
Stage I	128 (24.3%)	166 (31.5%)	
Stage II	65 (12.3%)	58 (11%)	
Stage III	55 (10.4%)	29 (5.5%)	
Stage IV	16 (3%)	10 (1.9%)	
Primary therapy outcome, n (%)			0.008
PD	47 (10.5%)	24 (5.4%)	
SD	19 (4.3%)	18 (4%)	
PR	2 (0.4%)	4 (0.9%)	
CR	149 (33.4%)	183 (41%)	
Gender, n (%)			0.154
Female	134 (25%)	152 (28.4%)	

Characteristic	Low expression of GRAP2	High expression of GRAP2	p
Male	133 (24.9%)	116 (21.7%)	
Age, n (%)			0.723
<=65	131 (25.4%)	124 (24%)	
>65	129 (25%)	132 (25.6%)	
Smoker, n (%)			0.443
No	34 (6.5%)	41 (7.9%)	
Yes	227 (43.6%)	219 (42%)	
Age, meidan (IQR)	65 (58, 72)	66.5 (59, 72)	0.591

Table2. Correlation analysis between GRAP2 expression and immune markers of immune cells from TIMER.

Description	Gene markers	None		Purity	
		Cor	P	Cor	P
CD8+ T cell	CD8A	0.727	***	0.674	***
	CD8B	0.646	***	0.593	***
T cell (general)	CD3D	0.728	***	0.654	***
	CD3E	0.81	***	0.768	***
	CD2	0.817	***	0.773	***
B cell	CD19	0.58	***	0.482	***
	CD79A	0.51	***	0.403	***
Monocyte	CD86	0.569	***	0.467	***
	CD115(CSF1R)	0.545	***	0.451	***
Neutrophils	CD66b(CEACAM8)	0.229	***	0.229	***
	CD11b(ITGAM)	0.473	***	0.382	***
	CCR7	0.669	***	0.578	***
Natural killer cell	KIR2DL1	0.285	***	0.238	***
	KIR2DL3	0.363	***	0.303	***
	KIR2DL4	0.317	***	0.253	***
	KIR3DL1	0.292	***	0.236	***
	KIR3DL2	0.393	***	0.335	***
	KIR3DL3	0.147	***	0.13	**
	KIR2DS4	0.323	***	0.269	***
Dendritic cell	HLA-DPB1	0.616	***	0.544	***
	HLA-DQB1	0.431	***	0.329	***
	HLA-DRA	0.568	***	0.483	***
	HLA-DPA1	0.594	***	0.525	***
	BDCA-1(CD1C)	0.376	***	0.29	***
	BDCA-4(NRP1)	0.204	***	0.169	***
	CD11c(ITGAX)	0.558	***	0.469	***
Th1	T-bet(TBX21)	0.73	***	0.677	***

	STAT4	0.624	***	0.544	***
	STAT1	0.478	***	0.405	***
	IFN-γ(IFNG)	0.56	***	0.499	***
	TFN-α(TFN)	0.42	***	0.296	***
Th2	GATA3	0.492	***	0.393	***
	STAT6	0.2	***	0.236	***
	STAT5A	0.658	***	0.592	***
	IL13	0.28	***	0.219	***
Tfh	BCL6	0.066	***	0.071	
	IL21	0.408	***	0.374	***
Th17	STAT3	0.113	*	0.137	**
	IL17A	0.33	***	0.271	***
Treg	FOXP3	0.652	***	0.562	***
	CCR8	0.653	***	0.569	***
	STAT5B	0.469	***	0.478	***
	TGFβ(TGFB1)	0.38	***	0.291	***
Effector T-cell	CX3CR1	0.381	***	0.324	***
	FGFBP2	0.327	***	0.278	***
	FCGR3A	0.483	***	0.394	***
Effector memory T-cell	DUSP4	-0.023		-0.032	
	GZMK	0.737	***	0.673	***
	GZMA	0.681	***	0.613	***
Resident memory T-cell	CD69	0.683	***	0.617	***
	CXCR6	0.78	***	0.729	***
	MYADM	0.181	***	0.079	
General memory T-cell	CCR7	0.669	***	0.578	***
	SELL	0.621	***	0.527	***
	IL7R	0.633	***	0.537	***
Exhausted T-cell	HAVCR2	0.559	***	0.456	***

	LAG3	0.576	***	0.509	***
	CXCL13	0.092	*	-0.016	
	LAYN	0.281	***	0.14	**

3.2 Low GRAP2 expression is an independent prognostic factor for overall survival in LUAD

We investigated whether GRAP2 expression correlates with prognosis in patients with LUAD. We divided the LUAD patients from the TCGA database into high (top 50% samples) and low (50% remaining samples) cohorts according to GRAP2 expression level. LUAD patients with higher expression of GRAP2 expression exhibited good overall survival (OS) (HR=0.61, P=0.001) according to the Kaplan-Meier survival analysis (Figure 3A). However, the correlation between GRAP2 expression and the OS in LUSC was not significant (HR=0.95, P=0.727) (Supplementary Figure 1C). Therefore, we will only analyze the role of GRAP2 in LUAD in the follow-up. Subgroup analysis showed that high GRAP2 expression was significantly associated with longer OS in LUAD under the following features: T2 stage (HR = 0.66, P = 0.035), N0&N1 stage (HR = 0.67, P = 0.018), M0 stage (HR = 0.54, P = 0.001), Pathological stage III (HR = 0.55, P = 0.048), Primary therapy outcome, PD&SD (HR = 0.47, P = 0.008), Residual tumor R0 (HR = 0.54, P = 0.001), Smoker (HR = 0.61, P = 0.004), Male patients (HR=0.63, P=0.034), Female patients (HR=0.59, P=0.012), Age >65 years (HR=0.57, P=0.007) and Age <=65 years (HR=0.62, P=0.003) (Figure 3B-3L). Cox analysis was also used to explore the correlation between GRAP2 expression and OS. High GRAP2 expression was significantly associated with longer OS (Univariate Cox: hazard ratio HR = 602, 95% CI = 0.448–0.808, P <0.001; Multivariate Cox: hazard ratio HR = 602, 95% CI = 0.448–0.808, P <0.001) (Figure 4). Finally, we used an independent external GEO data set GSE37745 to verify our results, the results were consistent with those above, LUAD patients with higher expression of GRAP2 showed higher OS compared with patients with lower expression of GRAP2 (Supplementary Figure 2). These data indicate that GRAP2 is a tumor suppressor gene and can be used as an independent prognostic factor for OS in LUAD.

3.3 GRAP2 is associated with immune response in LUAD

To examine the biological function of GRAP2 in LUAD, we used the GEPIA database to detect the co-expression pattern of GRAP2 in LUAD. The first 25 genes (top 25) are positively correlated with GRAP2 expression, and the last 25 genes (bottom 25) are negatively correlated with GRAP2 expression in the heat map (Figure 5A). We used the “Link Interpreter” module of the Linked Omics website to evaluated functional enrichment in the genes co-expressed with GRAP2 (top 600) using GO and KEGG annotations, and found that these genes were enriched in immune response processes, such as Th17 cell differentiation, T cell activation, initial immune deficiency, cytokine receptor activation and so on (Figure 5B). We also performed GO and KEGG annotations analysis in LUSC. The data showed that enriched pathways in the genes co-expressed with GRAP2 (top 600) were closely associated with the immune

response in LUSC, but there are few overlapping enrichment items between LUAD and LUSC (Supplementary Figure 3).

To gain further insight into the underlying mechanisms that GRAP2 regulates LUAD prognosis. The survival-related and down-regulated genes in LUAD were screened using GEPIA database. We crossed the 600 genes that co-expressed with GRAP2 with 731 survival-related and down-regulated genes in LUAD, we identified a gene-set containing 91 genes at the intersection (Figure 5C). We performed functional enrichment in these 91 genes using GO and KEGG annotations, several biological processes appeared as particularly enriched, including external side of plasma membrane, specific granule membrane, MHC protein complex, T cell activation and lymphocyte differentiation (Figure 5D).

We further used protein-protein interaction (PPI) and correlation analysis to explore the interactions between these 91 proteins. We found a higher enriched protein–protein interaction network among these proteins than random proteins (Figure 6A). Furthermore, most of these proteins in the network have a strong positive correlation with each other (Figure 6B). Therefore, these established genes co-expressed with GRAP2 particularly related to immune response, which may be the molecular mechanisms that GRAP2 affecting the prognosis of patients with LUAD, and can be used as multi-gene biomarkers to predict the survival in LUAD.

3.4 The expression level of GRAP2 correlate with the immune infiltration in LUAD

Since the hub genes were enriched in immune response-related pathways, the association between the immune score and GRAP2 expression was further explored. We divided the cases into two cohorts according to the expression level of GRAP2 and estimated the immune score by the ESTIMATE database. The immune score in cohort with high GRAP2 expression was significantly higher than in low GRAP2 expression cohort (Figure 7A).

Researches show that the survival time of several cancer patients is determined by the numbers and activity status of TIICs [6]. Therefore, we used the TIMER database to explore the correlation between GRAP2 expression and immune infiltration in LUAD. The results showed that numerous immune cells (except γδ T cells and Th2 cells) showed higher immune infiltration level in the cohort with high GRAP2 expression (Figure 7B). Furthermore, GRAP2 expression level was negatively correlated with tumor purity, however, it was positively correlated with infiltration levels of B cells ($r=0.579$, $P<0.001$), $CD8^+$ T cells ($r= 0.512$, $P<0.001$), $CD4^+$ T cells ($r= 0.562$, $P <0.001$), macrophages ($r =0.252$, PP<0.001), neutrophils ($r=0.484$, $P<0.001$) and dendritic cells ($r =0.536$, $P<0.001$) (Figure 7C). The correlation between GRAP2 expression and immune cell survival in LUAD was also examined. The data showed that cohort with high GRAP2 expression had a higher cumulative survival time in B cells ($P=0$) and dendritic cells ($P=0.048$), but not in $CD8^+$ T cells, $CD4^+$ T cells, neutrophils and macrophages (Figure 7D). These data suggest that GRAP2 plays a specific role in immune infiltration in LUAD.

3.5 GRAP2 is positively correlated with various immune markers

In order to further explore the relationship between GRAP2 and immune response, TIMER database was used to investigate the correlations between GRAP2 expression and diverse immune markers in LUAD. These immune markers can be used to characterize immune cells, including T cell (general), CD8+ T cell, Th1, Th2, Follicular helper T cell, Th17, Treg, Effector T-cell, Effector memory T-cell, Resident memory T-cell, General memory T-cell, Exhausted T-cell, B cell, Monocyte, Neutrophils, Natural killer cell and Dendritic cells. In clinical cancer biopsies, tumor purity is an important parameter to reflect the level of immune infiltration [23]. Our data showed that GRAP2 expression was positively correlated with most immune markers in multiple types of immune cells, 58 of 59 immune markers presenting a positive correlation with GRAP2 expression in LUAD (Table 2). These data further support that GRAP2 expression is significantly correlated with immune infiltration.

3.6 GRAP2 is positively correlated with chemokines/ chemokines receptors and MHC molecules

Chemokines and chemokine receptors play important role in the infiltration of immune cells towards the tumor [24]. To investigate the potential mechanism that GRAP2 regulating immune infiltration in LUAD, TISIDB database was used to examine the correlation between the expression level of GRAP2 and chemokines/ chemokine receptors. The heat map showed that GRAP2 expression was positively correlated with various chemokines and chemokine receptors in multiple tumors (Figure 8A, 8C). We also comprehensively explored the correlation between GRAP2 expression and chemokine/chemokine receptors using scatter plots. Data showed that GRAP2 expression was positively correlated with multiple chemokines, such as CCL4, CCL5, CCL18, CCL19, CXCL9, CXCL10, CXCL11, CXCL13 and XCL2 (Figure 8B). GRAP2 expression level was also positively associated with multiple chemokine receptors, such as CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, CXCR4, and CXCR6 (Figure 8D).

A growing body of research has shown that downregulation of major histocompatibility complex class-I and -II (MHC-I and MHC-II) promote tumor immune suppression, metastatic progression and a poor prognosis in multiple tumors [25, 26]. Therefore, TISIDB database was used to explore the correlation between GRAP2 expression and MHC molecules. The heat map results showed that the MHC molecules in numerous tumors were significantly positively correlated with the expression of GRAP2 (Figure 9A). Scatter plots showed that the MHC molecules were positively correlated with the expression of GRAP2, including B2M, HLA-B, HLA-C, HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DPB1, HLA-DOA1, HLA-DOA2, HLA-DRA, HLA-DRB1, HLA-E, HLA-F and TAP1 (Figure 9B-9Q). These data suggest that GRAP2 participating widely in modulating multiple immune-related molecules in LUAD to affect immune infiltration and immune response in TME.

4. Discussion

GRAP2 is an adaptor protein of the GRB2 family [8, 11]. Studies have shown that GRAP2 plays an important role in the development and function of T cells [9]. GRAP2 needs to form a complex with other proteins to trigger activation of downstream signaling molecules, SLP-76 is a common partner. GADS/SLP-76-mediated complexes at LAT (linker for the activation of T cells) activate multiple signaling pathways, including cytoskeleton rearrangement and adhesion, calcium signaling, and cell proliferation in T cells [12]. At present, the role of GRAP2 in tumors is unclear. In this study, TCGA, TIMER and TISIDB public databases were used to perform bioinformatics analysis, GRAP2 gene showed significantly lower expression than in adjacent normal tissue in LUAD. In the prognostic analysis, we found that the down-regulation of GRAP2 was associated with poor OS. At the same time, low expression of GRAP2 was associated with poor clinicopathological characteristics. These findings indicate that GRAP2 can be used as a tumor suppressor gene and prognostic biomarker. In order to explore the molecular mechanism underlying GRAP2 affecting the prognosis of patients with LUAD, we identified 91 genes from the co-expressed genes with GRAP2 as our hub genes, which can predict the prognosis and pathological stage of patients with LUAD. The prognostic value of GRAP2 in LUSC was also examined. We found that GRAP2 expression is down-regulated in LUSC, but had no association with OS. Although the expression of GRAP2 is down-regulated in both of LUAD and LUSC, the function of GRAP2 may be different in each other. Our GO and KEGG analysis show that a few overlapping enrichment items in between LUAD and LUSC. Nevertheless, GRAP2 is closely associated with the signaling pathways related to immune response in both LUAD and LUSC.

Cancer cells have evolved multiple mechanisms to evade immune attack, including the dysfunction of tumor antigen presentation and the recruitment of immunosuppressive cells [27–29]. Increased studies have demonstrated that different populations of immune cells showed distinct effects on tumor progression and oncotherapy [30–32]. Reports show that $CD8^+$ T cells, NK cells and perhaps NKT cells may mediate an anti-tumor immunity, whereas Treg cells, myeloid-derived suppressor cells, Th22 cells and perhaps B cells may promote tumorigenesis [24, 33, 34]. Here, we investigated the correlation between GRAP2 expression and immune infiltration in LUAD. We found that GRAP2 expression was positively correlated with the infiltration of a large number of immune cells, and a majority of immune marker sets of various immune cells. These results indicate that GRAP2 is associated with recruitment of immune cell into the TME both anti-tumor subsets and pro-tumor subsets. So, the precise role of GRAP2 in TME still needs further exploration.

Chemokines are secreted proteins with low molecular weight that are mainly mediate immune cell trafficking and lymphoid tissue development [24]. Immune cells are recruited into the TME by interactions between chemokines and paired chemokine receptors [6, 35]. In this study, we detected the correlation between GRAP2 expression and chemokines and chemokine receptors in LUAD. GRAP2 expression is significantly correlated with various chemokines and chemokine receptors. These may be explained how GRAP2 regulates immune infiltration in LUAD.

MHC molecules participate in antigen recognition in immune response [36]. Simple antigens cannot activate immune cells. Antigens are degraded by cytosolic and nuclear proteasomes and bound to

antigen-binding sites of MHC molecules on the surface of antigen presenting cells that can be recognized by T and B cells [37]. The expression of MHC on the surface of tumor cells represents the characteristics of tumor cells [38]. The poorer the differentiation of tumor cells, the weaker the expression of MHC molecules, which would result in immune escape of tumor cells [39, 40]. Here our data indicated that GRAP2 expression was positively correlated with numerous MHC molecules. These data strongly suggest that GRAP2 plays an important role in the presentation of tumor antigens in LUAD.

Conclusion

We conclude that GRAP2 is a tumor suppressor gene and can be used as a possible prognostic marker in LUAD. Decreased expression of GRAP2 is correlated with poor clinical characteristics and low immune infiltration. Therefore, we can try to assess the degree of malignancy of LUAD by detecting the expression level of GRAP2 in the surgical specimens of LUAD, and even better evaluate the status of TME.

Abbreviations

LUAD
Lung adenocarcinoma
LUSC
Lung squamous cell carcinoma
GEO
Gene Expression Omnibus
TME
tumor microenvironment
TIICs
tumor infiltrating immune cells
HR
hazard ratio
GEPIA
Gene Expression Profiling Interactive Analysis
IHC
Immunohistochemistry
GO
Gene ontology
KEGG
Kyoto encyclopedia of genes and genomes
PPI
Protein–protein interaction
MHC
Major histocompatibility complex

OS
Overall survival
FPS
First-progression survival.

Declarations

Statements

We statement that all methods were performed in accordance with the relevant guidelines and regulations.

Ethics approval and consent to participate

The study involving human participants were reviewed and approved by The Institutional Research Ethics Committee of Taihe Hospital (authorization number: 2021KS036). The patients/participants provided their written informed consent to participate in this study.

Consent for publication

All authors agreed to the publication of the manuscript.

Data availability statement

The raw data for this article can be found from https://cancergenome.nih.gov, and https://www.ncbi.n1m.nih.gov/gds/?term=. Further inquiries can be directed to the corresponding author.

Competing interests

The authors declare that there is no conflict of interest in this work.

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Author contributions

Study concept and design: ZL and YY. Acquisition of data: SS, XD, and JW. Analysis and interpretation of data: JH, ZP, YL and SJ. Statistical analysis: MS, JH and ZP. Drafting of the manuscript: SS and XD. Obtained funding: XD, YY and ZL. Study supervision: YY, SJ and ZL. All authors contributed to the article and approved the submitted version.

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Not applicable.

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Figures

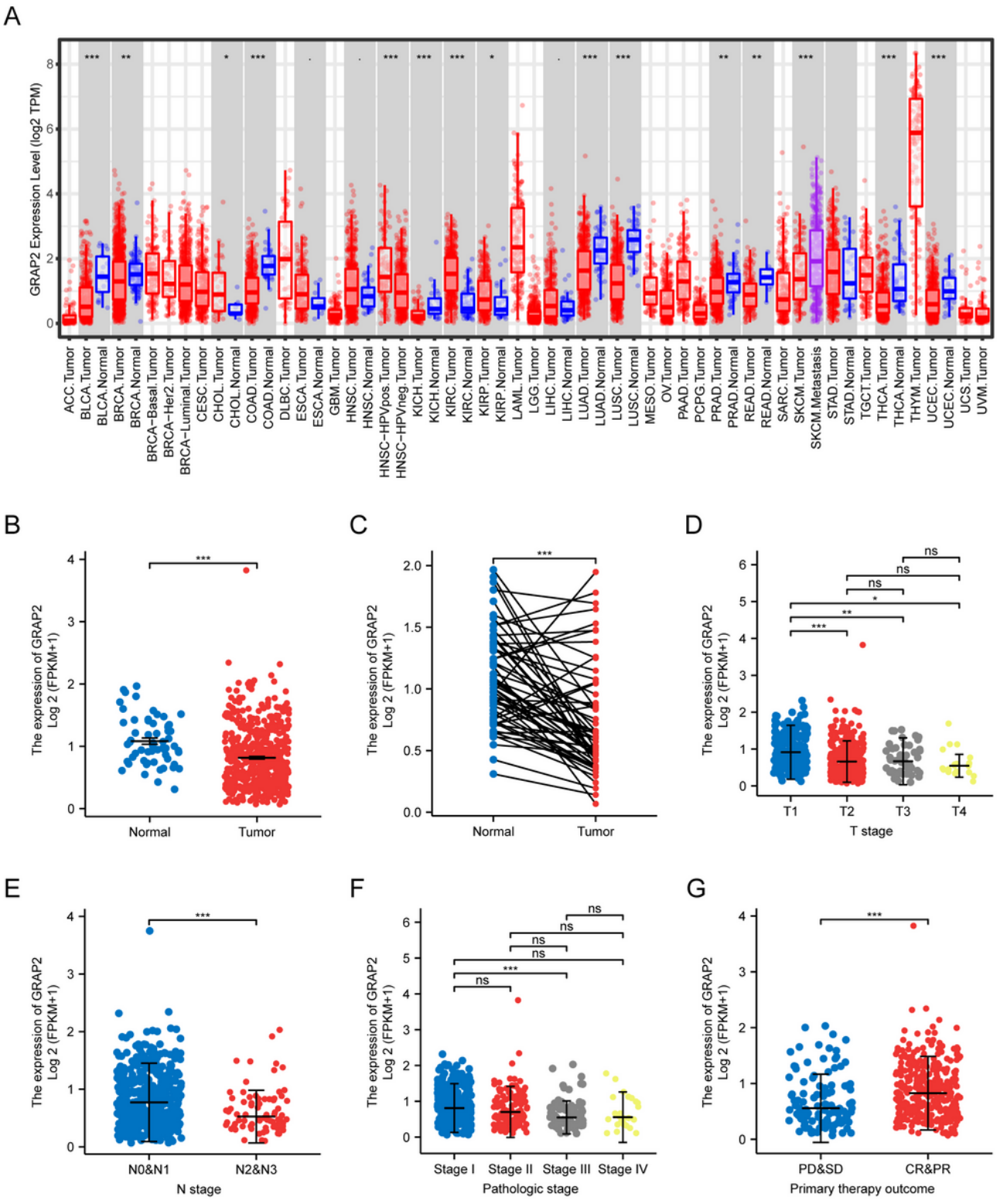

Figure 1

The mRNA levels of GRAP2 in different human cancers. (A) Using TIMER to detect mRNA expression levels of GRAP2 in different tumors. The mRNA expression level of GRAP2 in cancer tissues and adjacent normal tissues (unmatched tissues) (B), (matched tissues) (C), and the cancer tissues from patients with different clinical features in TCGA, (D) T stage, (E) N stage, (F) pathologic stage, (G) primary therapy outcome.

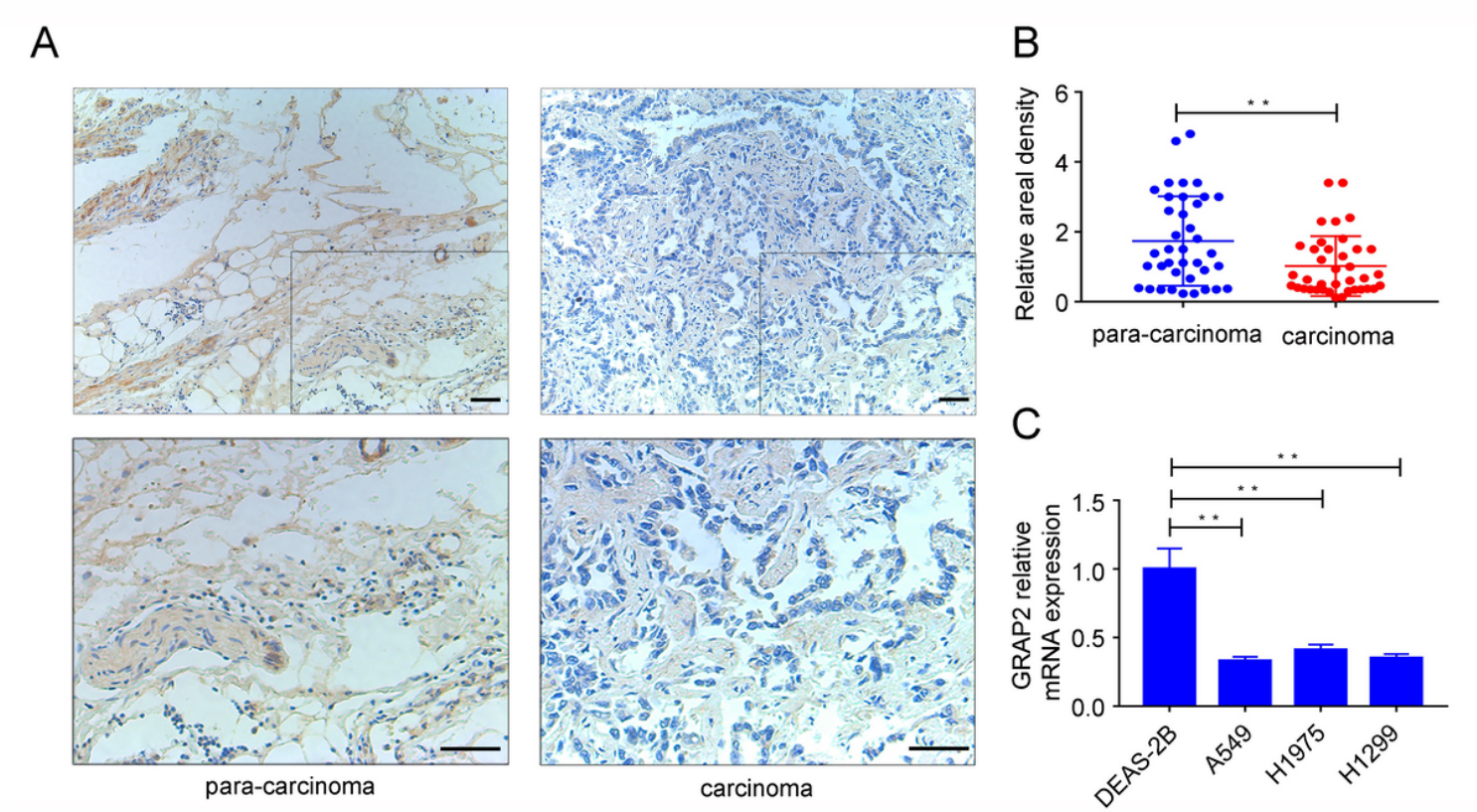

Figure 2

Protein expression levels of GRAP2 in patients with lung cancer. (A) GRAP2 expression levels in cancer tissues and adjacent normal tissues were detected by immunohistochemical staining. Scare bars, 50 μm. (B) Statistical graph for immunohistochemical staining. The dot plot shows the means and standard deviation of 76 images of cancer tissues and adjacent normal tissues. (C) GRAP2 expression in four different cell lines was detected by real-time PCR.

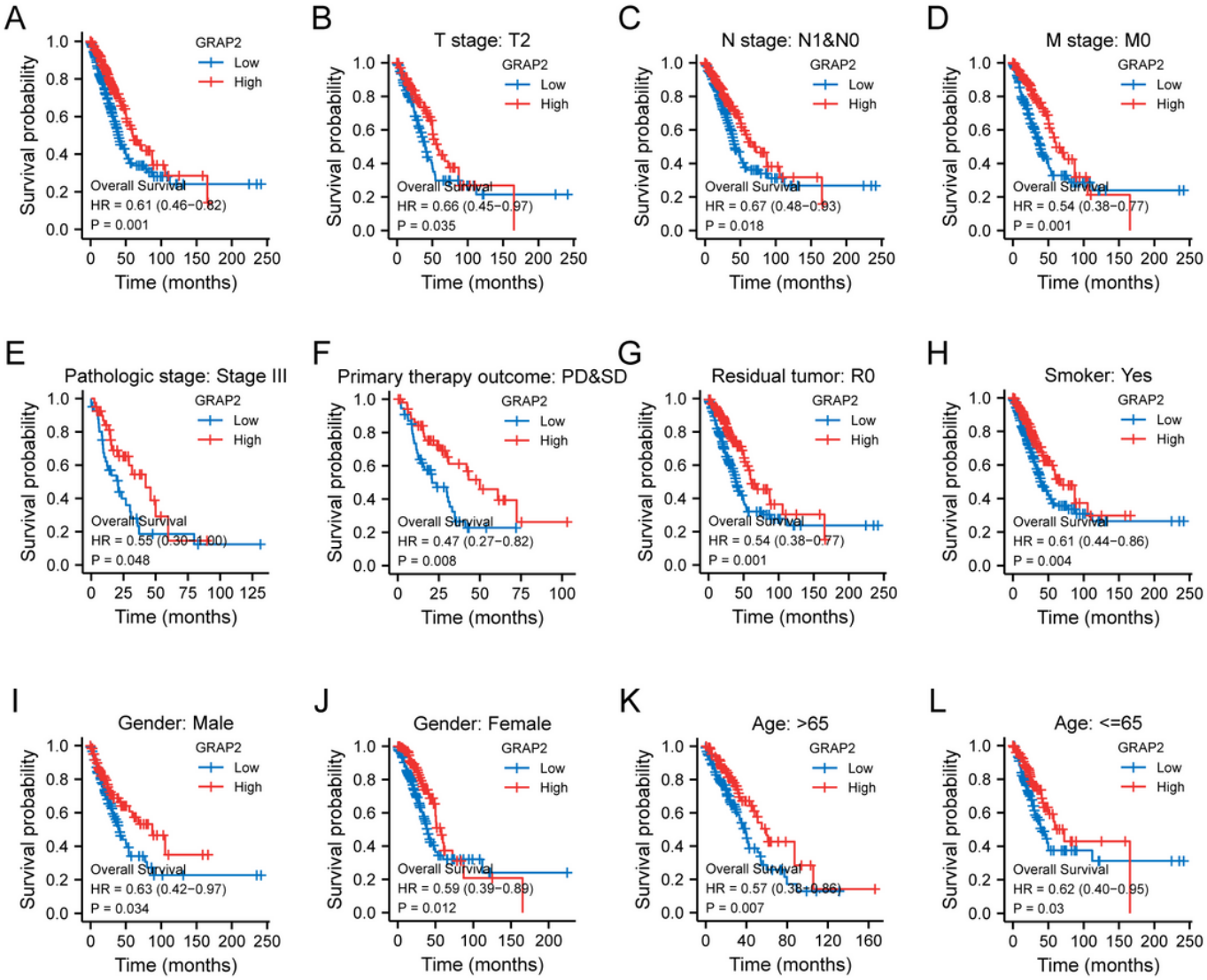

Figure 3

Kaplan-Meier overall survival of GRAP2 in LUAD. (A) Kaplan-Meier estimates the effect of GRAP2 on OS in LUAD. (B-L) Subgroup analysis for T1, N0 & N1, M0, Pathological staging stage III, PD & SD, Residual tumor stage R0, smoker, Male, Female, age>60, age<=60.

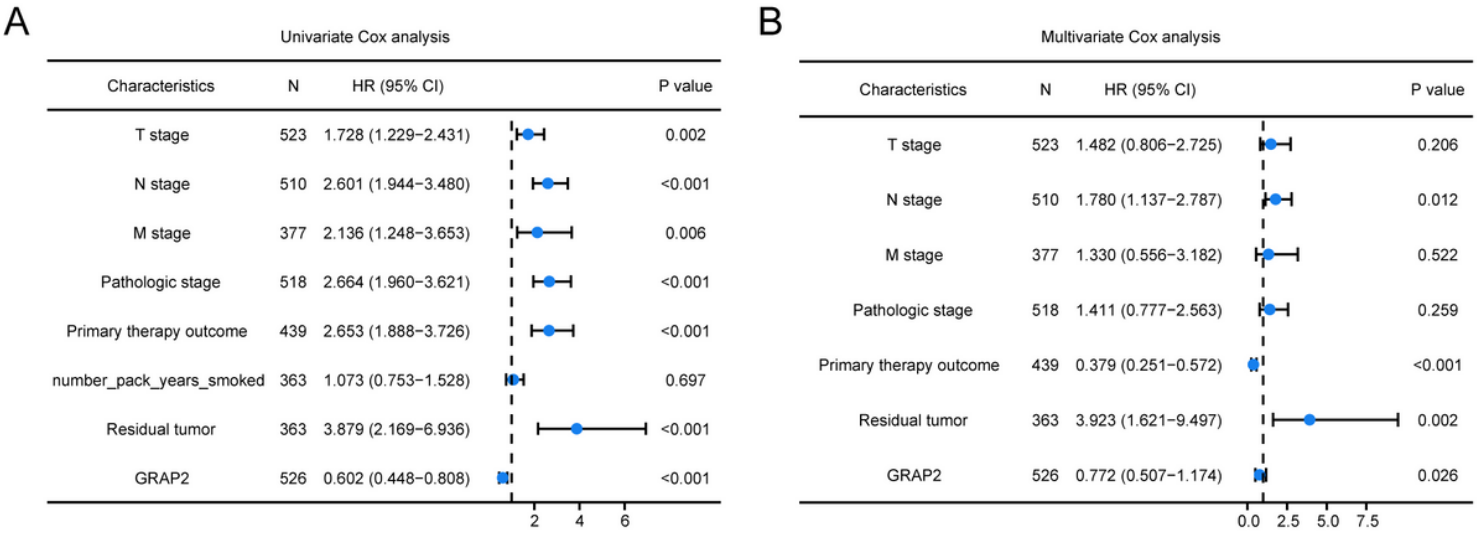

Figure 4

Forest plot of the univariate and multivariate cox regression analysis in LUAD. (A) Univariate and (B) multivariate cox regression analyses of risk score and other clinical characteristics in the TCGA data set.

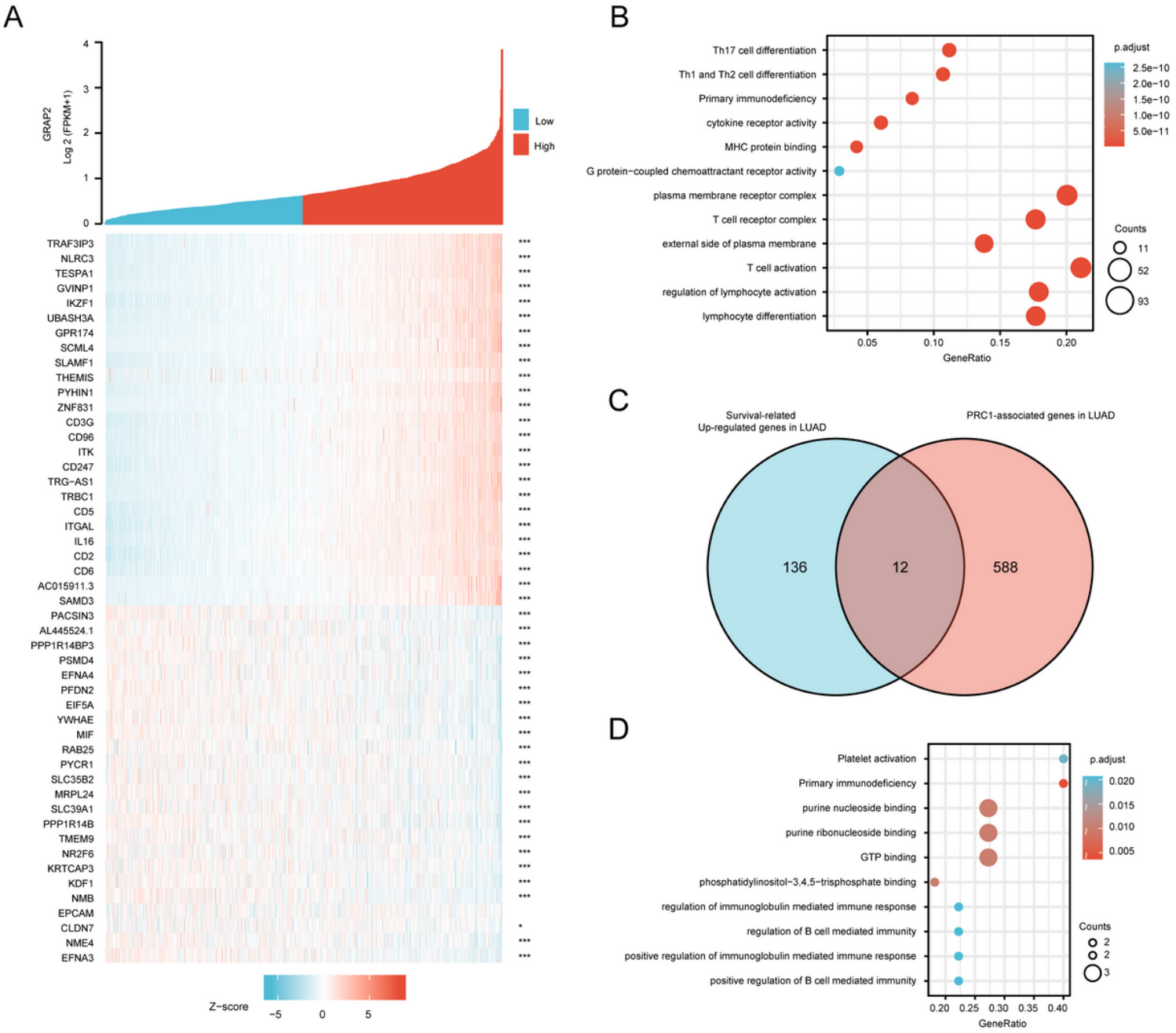

Figure 5

GRAP2 functional clustering and interaction network analysis of co-expressed genes with GRAP2. (A) The heat map shows the top 50 genes in LUAD that are positively or negatively correlated with GRAP2. (B) GO term and KEGG pathway analysis of co-expressed genes of GRAP2 in LUAD. (C) Venn diagram of co-expressed genes of GRAP2 and survival-related and down-regulated genes in LUAD. (D) GO and KEGG analysis of intersection between co-expressed genes of GRAP2 and survival-related genes in LUAD.

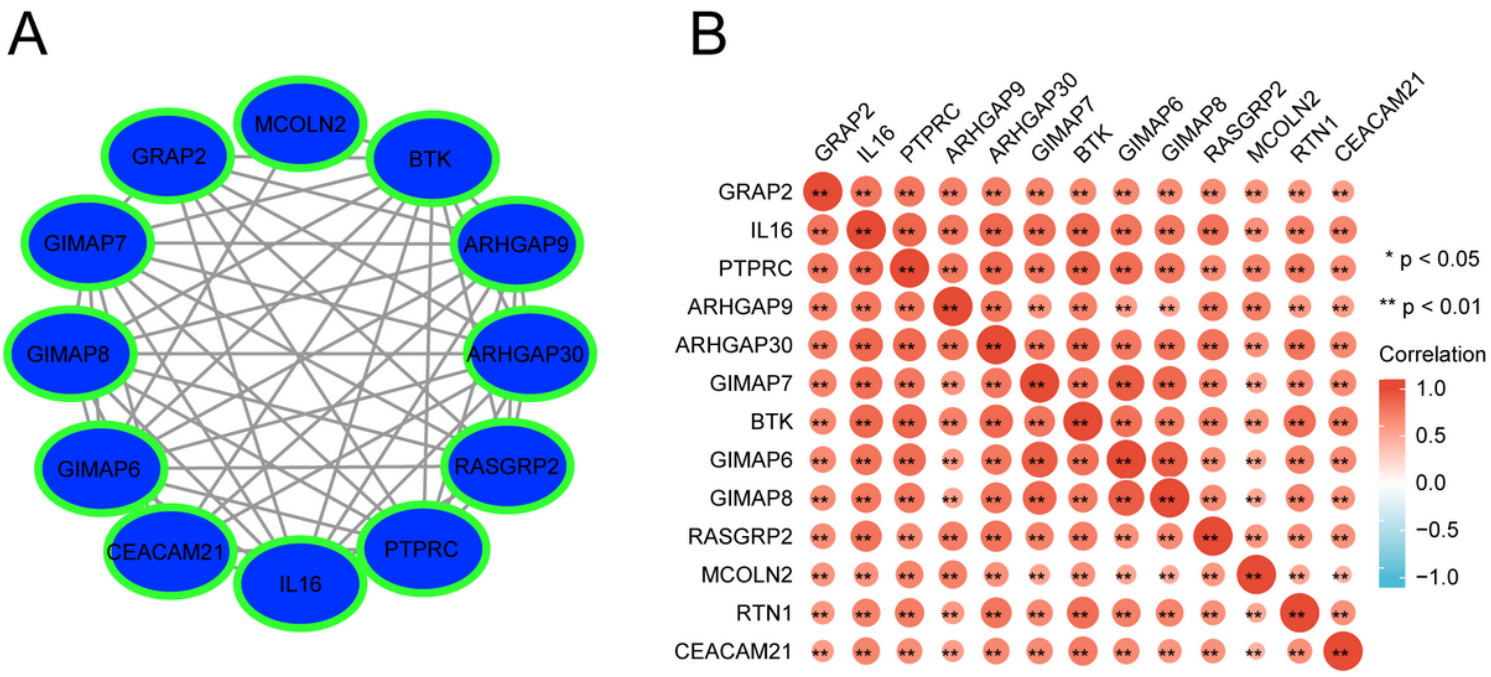

Figure 6

Interaction analysis. (A) Interaction network and (B) gene co-expression matrix of the co-expressed genes with GRAP2.

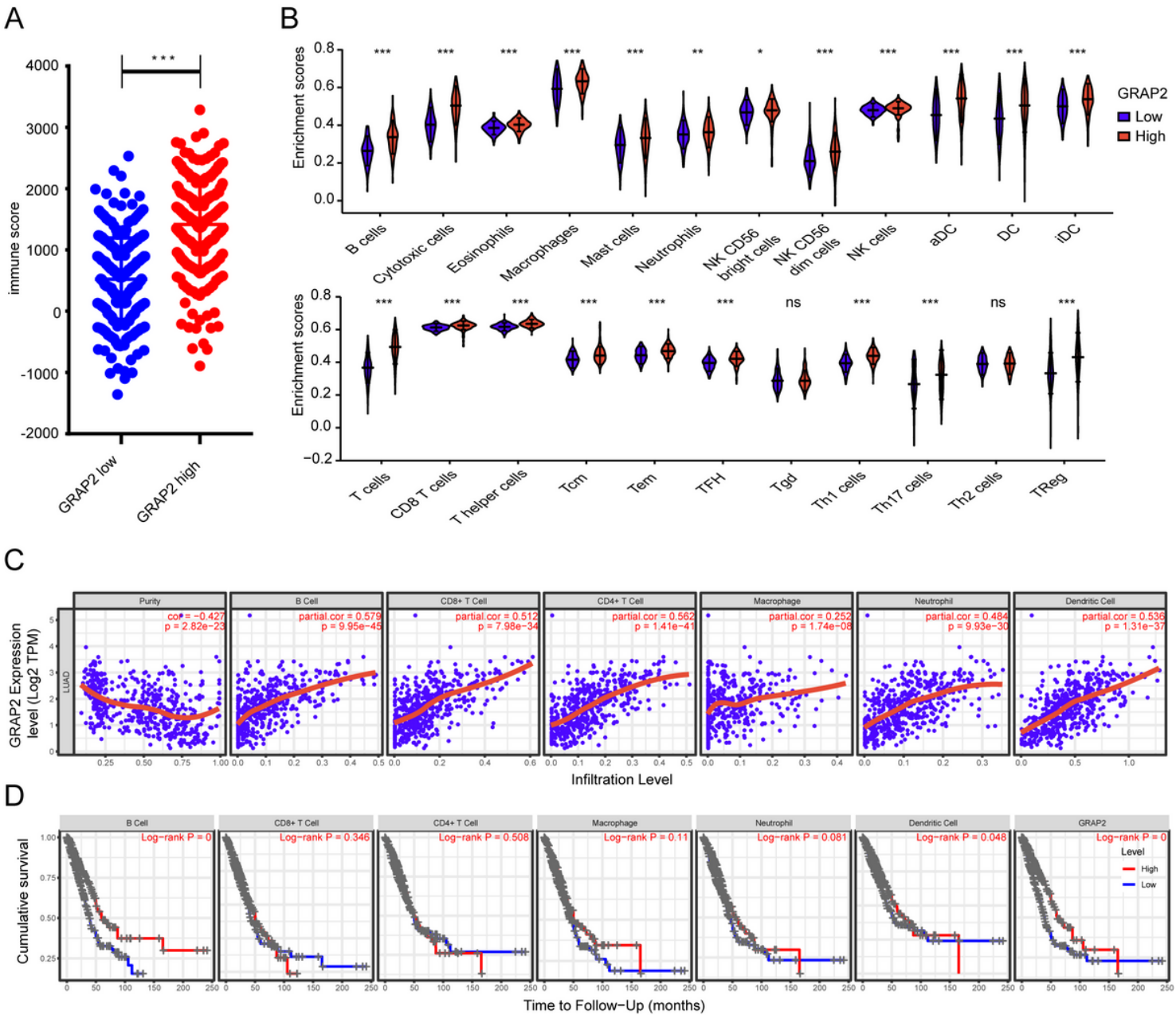

Figure 7

Correlation analysis of GRAP2 expression and immune infiltration in LUAD. (A)The correlation between immune score and GRAP2 expression by ESTIMATE database.(B)The distribution of immune cells in cohorts with high GRAP2 expression or low GRAP2 expression. The correlation between GRAP2 expression (C) and immune infiltration and cumulative survival time (D) in B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells.

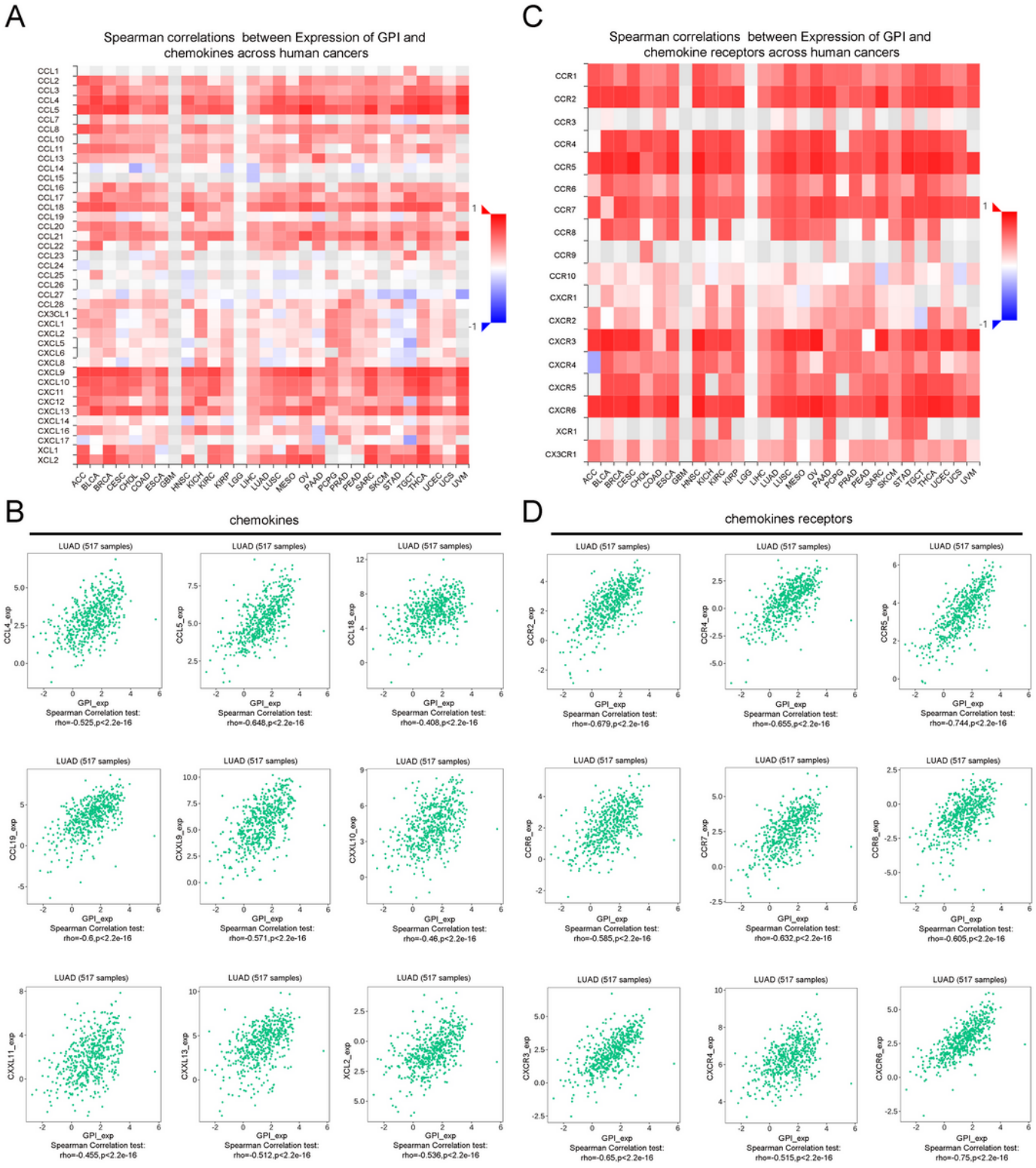

Figure 8

Correlation analysis between GRAP2 expression and chemokines and /chemokine receptors. (A & C) Heat map analysis of the correlation between GRAP2 expression and chemokines /chemokine receptors in tumors. (B & D) Scatter diagram analysis for the correlation between GRAP2 expression and chemokines /chemokine receptors in LUAD.

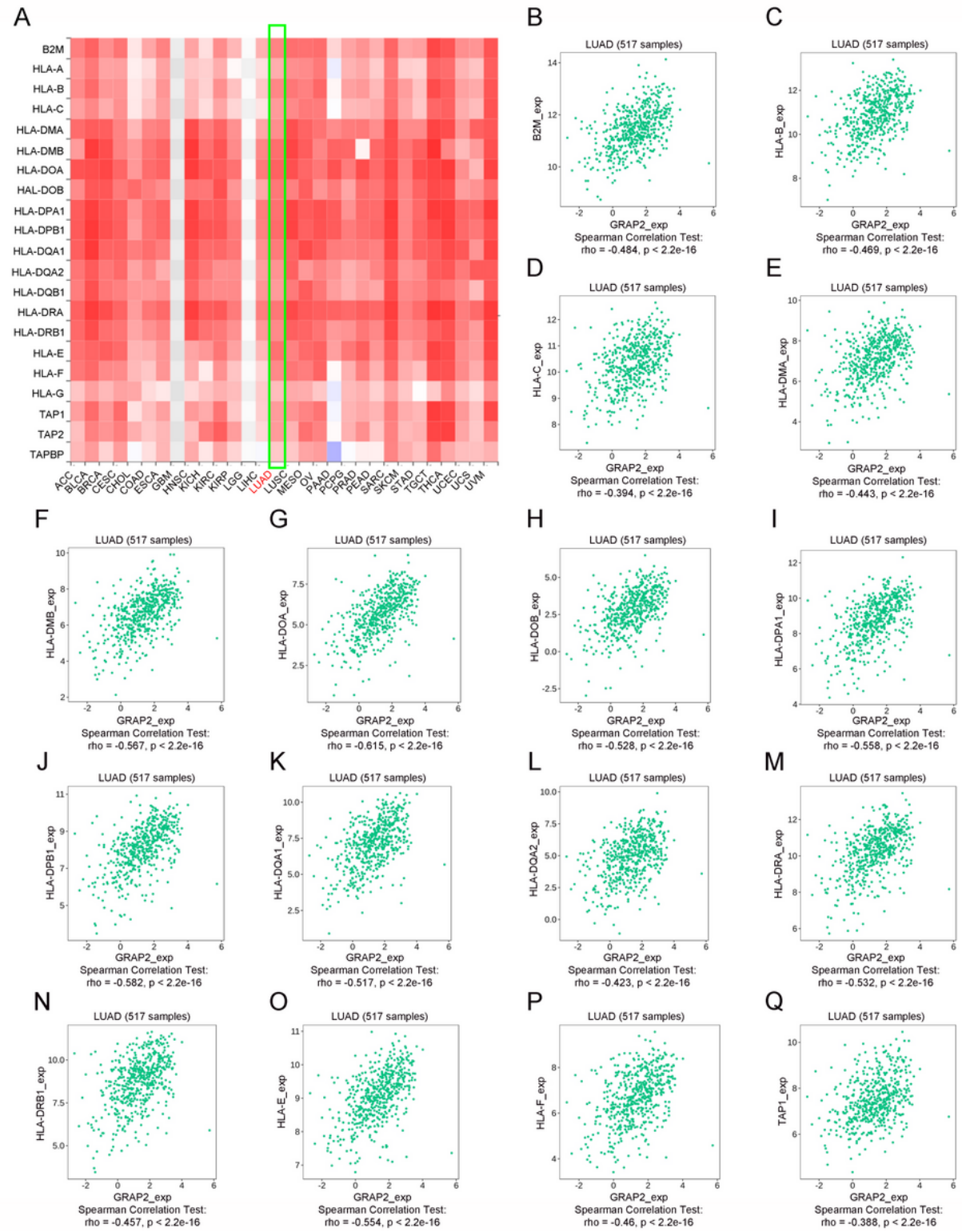

Figure 9

Correlation analysis between GRAP2 expression and MHC molecules. (A) Correlation between GRAP2 expression and MHC molecules in tumors by heat map analysis. (B-Q) Correlation between GRAP2 expression and MHC molecules in LUAD by scatter diagram analysis.

Supplementary Files

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- SupplementaryMaterial.docx