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-specific protein-tyrosine kinase signaling; however, the prognostic value of GRAP2 and its correlation with immune cell infiltration in lung adenocarcinoma (LUAD) remain unclear.

Methods: All original data were downloaded from the TCGA database and integrated via R 3.2.2. GRAP2 expression was explored with the TCGA and TIMER databases. We evaluated the influence of GRAP2 on clinical prognosis using the Kaplan-Meier plotter, Gene Expression Omnibus (GEO) database and GEPIA database. Correlations between GRAP2 and cancer immune characteristics were analyzed via TIMER and TISIDB databases. 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 identified a hub gene that included a total of 91 GRAP2 co-expressed genes, which were tightly associated with immune response in LUAD. GRAP2 expression was 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 level 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 determining prognosis and immune infiltration in LUAD.

1. Introduction

Lung cancer is one of the malignant tumors with the highest incidence and the worst prognosis in the world [1]. Lung cancer is divided into small cell lung cancer and non-small cell lung cancer (NSCLC). Lung adenocarcinoma (LUAD) is the largest subgroup of NSCLC [2, 3]. Despite improvements in systemic treatments for patients, the 5-year survival rate of LUAD patients is 15% [4]. 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 [5]. Increased evidences indicate that TIICs determine the success of immunotherapy and affects the prognosis of patients [6]. Increased evidences indicate that $CD8^+$ T cells, $CD4^+$ Th1 T cells and natural killer cells are critical players in eliminating malignant cells in the healthy human organism which has widely been demonstrated in mice

models where mice lacking these cells have a significantly higher probability to develop malignancies. On the contrary, B cells, M2-macrophages and Treg cells are deeply involved in promoting malignant progression [7, 8]. It is currently unclear which factors drive immune infiltration in LUAD. Therefore, it is necessary to find new biomarkers to identify immune infiltration in LUAD.

GRAP2 is an adaptor protein involved in leukocyte-specific protein-tyrosine kinase signal transduction. This protein shares 40 – 50% sequence homology in the SH3 and the SH2 domain with Grb2 protein [9]. 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 in T cells [10]. At present, research of GRAP2 mainly focuses on the immune system. 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 [11].

In recent years, more and more platforms and databases have enabled cancer researchers to use multiple sets of data for cancer bioinformatics analysis. In order to better understand the role of GRAP2 gene in LUAD, we comprehensively assessed the relationship between GRAP2 expression and the prognosis of LUAD by TCGA, TIMER, and Kaplan-Meier plotter database. In addition, we also studied the correlation between GRAP2 and immune infiltration by the TIMER and TISIDB databases. Our results show that GRAP2 can be used as a biomarker for prognosis and immune infiltration prediction in LUAD.

2. Materials And Methods

Patient data set

The mRNA expression data (including 535 LUAD samples and 59 adjacent normal samples) and clinical information are downloaded from the TCGA database (https://cancergenome.nih.gov) [12]. Gene Expression Omnibus (GEO) dataset GSE37745 (including 196 LUAD samples).

Survival analysis

Kaplan-Meier plotter (http://kmplot.com/analysis/) [13] and Gene Expression Profiling Interactive Analysis (GEPIA) (http:GEPIA cancer-pku.cn) [14] was used to estimate the correlation between GRAP2 expression and the survival rate of different clinical features in LUAD patients, and the hazard ratio (HR) and log-rank P-value of 95% confidence interval were calculated.

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

The GeneMANIA database (http://www.genemania.org) [15] was applied to construct the correlation heat map for the co-expressed genes of GRAP2. PPI data are extracted from the STRING database (https://string-db.org) based on protein interaction and signal pathways, and the network is constructed by Cytoscape 3.7.2.

GEPIA database analysis

GEPIA (http://gepia.cancer-pku.cn/index.html) [14] 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 identify genes that associated with LUAD prognosis.

Linked omics database analysis

The Linked Omics database (http://www.linkedomics.org) [16] contains 32 cancer types from the TCGA project and a total of 11158 patients with multi-omics data and clinical data. In this study, the Linked Omics database was used to explore the gene ontology biological process (GO_BP) and KEGG pathway.

Timer database analysis

TIMER (http://www.cistrome.shinyapps.io) [17] is a convenient and accurate online analysis tool that can explore the expression levels of genes in normal and tumor tissues in multiple tumors in the TCGA data set, TIMER determines the abundance of TIICs based on the statistical analysis of gene expression profiles. In this study, the TIMER database was used to analyze the expression levels of genes 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) [18] is a web portal for tumor and immune system interaction. In order to clarify the role of GRAP2 in immune response in cancer, we used the "Immunomodulator" module of the TISIDB database to analyze and evaluate the correlation between GRAP2 expression and MHC molecules. In order to further study the relationship between GRAP2 and chemokine/chemokine receptor expression, we evaluated the chemokine/chemokine receptor expression level of TIIC by the "chemokine" module.

Western blot

LUAD cells were lysed with RIPA lysis buffer supplemented with protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific, Shanghai, China). The protein lysate was separated by SDS-PAGE gel (Servicebio, Wuhan, China) and printed on PVDF membrane (Millipore, USA) for analysis [19]. Anti-GRAP2 (1:2000 dilution, Cambridge, MA, USA, ab224613) and anti-GAPDH (1:5000 dilution, Proteintech, China, 60004-1-Ig) were incubated overnight at 4°C. 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

This study was approved by the Institutional Research Ethics Committee of Taihe Hospital. Seventy-six paraffin-embedded lung adenocarcinoma tissues and para-carcinoma tissues were used for IHC staining.

Each tissue was incubated overnight with a primary antibody for GRAP2 (1:2000 dilution, MA, USA, ab224613) at 4°C. After washing with phosphate-buffered saline (PBS), each section was incubated with HRP-labeled goat anti-mouse secondary antibody (1:2000 dilution, Beyotime, China, A0181) for 2 h at room temperature. IHC staining was achieved using 3,3'-diaminobenzidine and counter-stained with hematoxylin (Beyotime). The IHC staining results were analyzed and scored by two pathologists who were blinded to the sources of the clinical samples. ImageJ software was used to analyze the intensity of staining by a semiquantitative integration method [20].

Statistical analysis

The results generated in TIMER were displayed with P-values, fold changes, and ranks. The critical value of GRAP2 expression was selected as the median method of gene expression. The results of Kaplan-Meier plots, and GEPIA were displayed with HR and P or Cox P-values from a log-rank test. 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. Statistical significance was determined using one-way ANOVA with the post hoc Tukey test. In all analyses, *, * *, and * * * indicate P <0.05, P <0.01, and P <0.001.

3. Results

3.1 Decreased GRAP2 expression in LUAD

In order to explore the expression level of GRAP2 in tumor tissues and normal tissues, we used the TIMER database to analyze the expression level of GRAP2 mRNA in different tumors. The results showed that, compared with normal tissues, GRAP2 expression levels in bladder urothelial carcinoma, breast invasive carcinoma, colon adenocarcinoma, LUAD, lung squamous cell carcinoma (LUSC), prostate adenocarcinoma, rectum adenocarcinoma and other tumor tissues were significantly lower than normal tissues. However, higher expression was observed in tumors such as esophageal carcinoma and kidney renal clear cell carcinoma (Figure 2A).

We further used the TCGA database to evaluate the mRNA expression level of GRAP2 in tumor tissues compared with adjacent normal tissues in LUAD. The results showed that the expression level of GRAP2 was significantly lower than that in unmatched adjacent normal tissues (P<0.01) (Figure 2B). These results were verified in matched tumor tissues and adjacent normal tissues (Figure 2C). We also found that the mRNA expression level of GRAP2 was significantly lower than that in adjacent normal tissues in LUSC (Supplementary Figure 1A and 1B).

To investigate the correlation between GRAP2 expression and clinical characteristics in LUAD patients, we analyzed the mRNA expression levels of GRAP2 in different clinical categories in the TCGA database. Table 1 summarizes the correlation between GRAP2 expression and clinical characteristics in LUAD. The results show that the low expression of GRAP2 is associated with late T stage, N stage, Pathologic stage, and worse Primary therapy outcome (Figure 2D-2G and Table 1). The protein expression level of GRAP2

was further investigated by IHC staining, and we found that the GRAP2 protein level was obviously decreased in LUAD tissues compared with adjacent normal tissues (Figures 2A and 2B). Moreover, we found that GRAP2 mRNA expression was significantly down-regulated in three LUAD cell lines (A549, H1975 and H1299) compared to that in a nonmalignant lung epithelial cell line (BEAS -2B) (Figure 2C).

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

We investigated whether GRAP2 expression correlates with prognosis in cancer patients. We divided the LUAD patients in the TCGA database into high (top 50% samples with the highest expression) and low (50% remaining samples) cohorts according to GRAP2 expression level for survival analysis. LUAD patients with higher expression of the 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. Data showed that high GRAP2 expression was significantly associated with longer overall survival (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 result showed that the overall survival of LUAD patients with high expression of GRAP2 were significantly higher than those of patients with low expression (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 are positively correlated with GRAP2 expression, and the last 25 genes are negatively correlated with GRAP2 expression in the heat map (Figure 5A).

We used the “Link Interpreter” module of the Linked Omics website to identify the GO functional enrichment and KEGG pathway in the co-expressed genes of GRAP2 (top 600), 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 have also performed GO functional enrichment and KEGG pathway analysis in LUSC. The data showed that GRAP2 was closely associated with the immune response-related pathways in LUSC, but there are few overlapping enrichment items between LUAD and LUSC (Supplementary Figure 3).

To better understand the relevance and underlying mechanisms of GRAP2 expression on the prognosis of LUAD. 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, and detected 91 genes at the intersection (Figure 5C). These 91 protein-coding genes may be the potential genetic biomarkers for LUAD patients. GO functional enrichment and KEGG pathway analysis were performed in these 91 genes, and the results showed that these genes were enriched in external side of plasma membrane, specific granule membrane, MHC protein complex, T cell activation and lymphocyte differentiation and so on (Figure 5D).

We further used protein-protein interaction (PPI) and correlation analysis to identify the interactions between these 91 proteins. We found that there is a stronger enrichment network between these proteins than random proteins, and these genes are particularly related to the immune response pathway (Figure 6A). Gene co-expression correlation analysis showed that most of the 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, and can be used as multi-gene biomarkers to predict the survival in LUAD.

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

Since the signature was composed of immune-related genes, the association between the immune score and GRAP2 expression was further explored. We divided the cases into high-expression and low-expression cohorts according to the expression level of GRAP2 and estimated the immune score by the ESTIMATE database. The results showed that the immune score in GRAP2 high expression cohort was significantly higher than that in low expression cohort (Figure 7A).

Researches show that the survival time of several cancer patients is determined by the number and activity status of TIICs [5]. Therefore, we used the TIMER database to analyze 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 GRAP2 high expression cohort (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). We also analyzed the correlation between GRAP2 expression level and immune cell survival in LUAD. The data showed that cohort of 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

To deepen our understanding of GRAP2 crosstalk with the immune response, TIMER database was used to investigate the correlations between GRAP2 expression and diverse immune signatures in LUAD. The

genes listed were 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. Tumor purity is an important aspect affecting the dissection of immune infiltration in clinical cancer biopsies. After adjusting for tumor purity, GRAP2 expression was significantly associated with most immune markers in divergent types of immune cells, the GRAP2 expression level was significantly correlated with 58 of 59 immune markers 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 are essential for the immune cells towards the tumors [21]. Therefore, we used the TISIDB database to analyze the correlation between the expression level of GRAP2 and immune cell related chemokines/ chemokine receptors in LUAD. The heat map results showed that GRAP2 expression was positively correlated with various chemokines and chemokine receptors in multiple tumors (Figure 8A, 8C). In order to further clarify the relationship between GRAP2 expression and immune cell migration, we comprehensively analyzed the correlation between GRAP2 expression and chemokine/chemokine receptors. The results show that GRAP2 expression was positively correlated with immune cell-related chemokines, such as CCL4, CCL5, CCL18, CCL19, CXCL9, CXCL10, CXCL11, CXCL13 and XCL2 (Figure 8B). GRAP2 expression was also positively associated with immune cell-related chemokine receptors, such as CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR3, CXCR4, and CXCR6 (Figure 8D).

A large number of studies have shown that downregulation of major histocompatibility complex class-I and -II (MHC-I and MHC-II) has been linked to immune suppression, metastatic progression and a poor prognosis in numerous tumors [22]. Therefore, we used the TISIDB database to analyze 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). The expression level of GRAP2 was positively correlated with the expression of multiple MHC molecules, 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). Therefore, it was confirmed that GRAP2 participating widely in modulating various immune molecules in LUAD to affect immune infiltration in TME.

4. Discussion

GRAP2 is an adaptor protein of the GRB2 family. Studies have shown that GRAP2 plays an important role in the development and function of T cells [10]. GRAP2 needs to form a complex with other proteins to perform function, SLP-76 is a common partner. In T cells, GADS/SLP-76-mediated complexes at LAT (linker for the activation of T cells) lead to activation of a variety of pathways, including cytoskeleton rearrangement and adhesion, transcription, calcium signaling, and cell proliferation [11]. 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, we found that the expression of GRAP2 in LUAD was significantly lower than that in adjacent normal tissue. In the prognostic analysis, we found that the down-regulation of GRAP2 expression 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 underlying molecular mechanism of GRAP2 affecting the prognosis in LUAD, we defined 91 genes from the co-expressed genes of GRAP2 as our hub genes, which can predict the survival rate and pathological stage of patients with LUAD. We also analyzed the expression and prognostic value of GRAP2 in LUSC. We found that GRAP2 expression is down-regulated in LUSC, but there is no significant correlation 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 there were few overlapping enrichment items in between LUAD and LUSC. Nevertheless, we still found that GRAP2 is closely associated with the immune response-related pathways in LUAD and LUSC.

Although an effective immune response can play an anti-tumor effect, cancer cells have evolved a variety of mechanisms to evade immune attack, including the dysfunction of tumor antigen presentation and the recruitment of immunosuppressive cells [23-25]. Increased studies have demonstrated that different populations of immune cell have distinct effects on tumor progression and therapeutic outcomes [26-28]. Reports show that CD8+ T cells, NK cells and perhaps NKT cells may mediate an anti-tumor immunity, whereas Treg cells, Th22 cells and perhaps B cells may promote tumorigenesis [21, 29, 30]. In this study, we explored the correlation between GRAP2 expression and immune infiltration in LUAD. Our results show 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 small, secreted proteins that are best known for their roles in mediating immune cell trafficking and lymphoid tissue development [21]. Different immune cell subsets are recruited into the TME via interactions between chemokines and chemokine receptors [5, 31]. In this study, we detected the correlation between GRAP2 expression and the expression of immune cell related chemokines and chemokine receptors in LUAD. The results showed that 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. Simple antigens cannot activate immune cells. Antigens are degraded by cytosolic and nuclear proteasomes and bound to MHC molecules on the surface of antigen presenting cells that can be recognized by T and B cells [32]. The expression of MHC on the surface of tumor cells represents the characteristics of tumor cells [33]. The poorer the differentiation of tumor cells, the weaker the expression of MHC molecules, which would result in immune escape of tumor cells [34, 35]. Here our results showed 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, in clinical work, we can try to assess the degree of malignancy of the patient by measuring the expression of GRAP2 in the surgical specimens of LUAD patients, and even better evaluate the status of TME, and develop immunotherapeutic drugs targeting GRAP2.

Declarations

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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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Data availability statement

The Supplementary Material for this article can be found online. Further inquiries can be directed to the corresponding author.

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.

Competing interests

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

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Tables

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%)	
Male	133 (24.9%)	116 (21.7%)	
Age, n (%)			0.723

Characteristic	Low expression of GRAP2	High expression of GRAP2	p
<=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	**

Figures

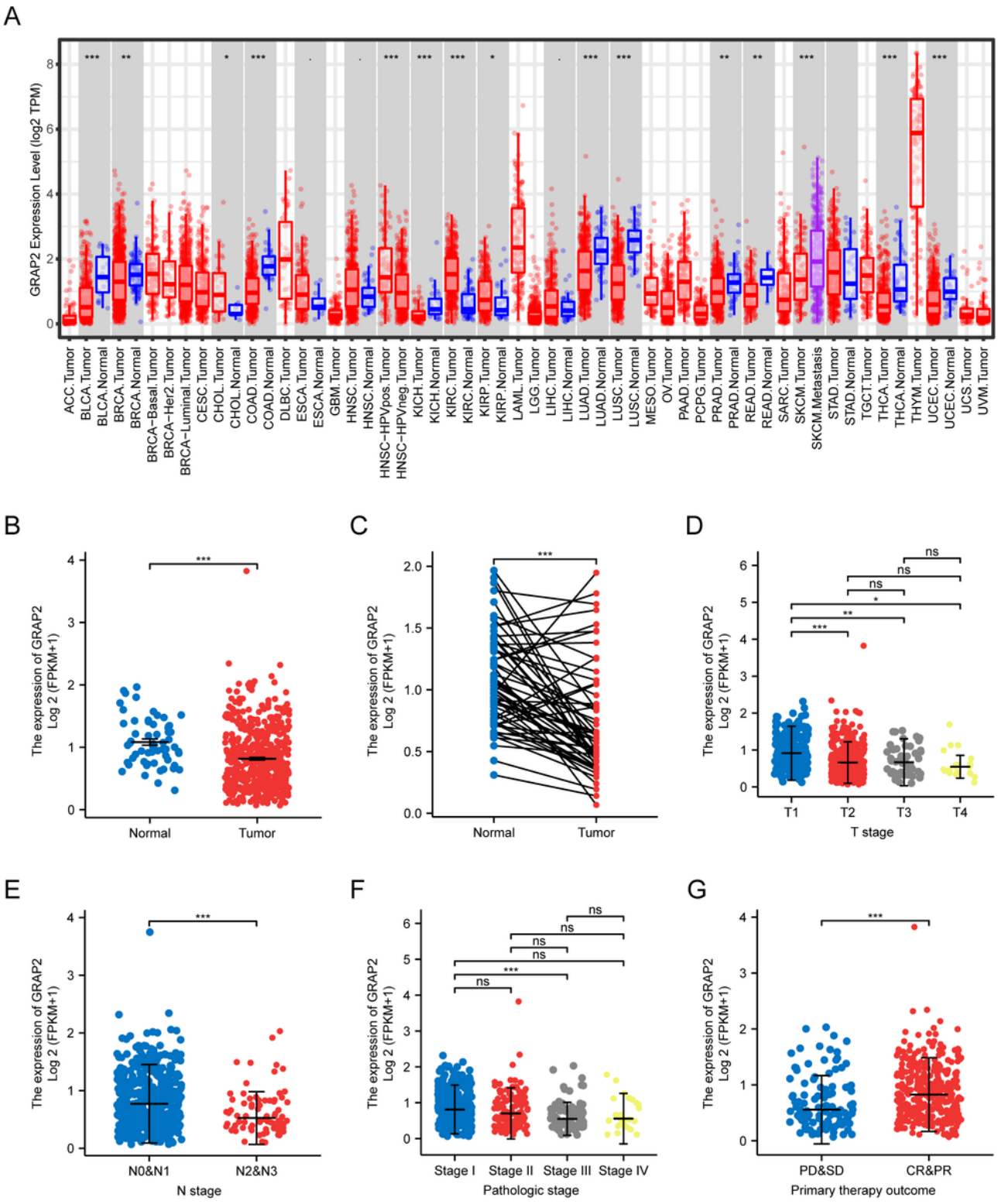

Figure 1

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

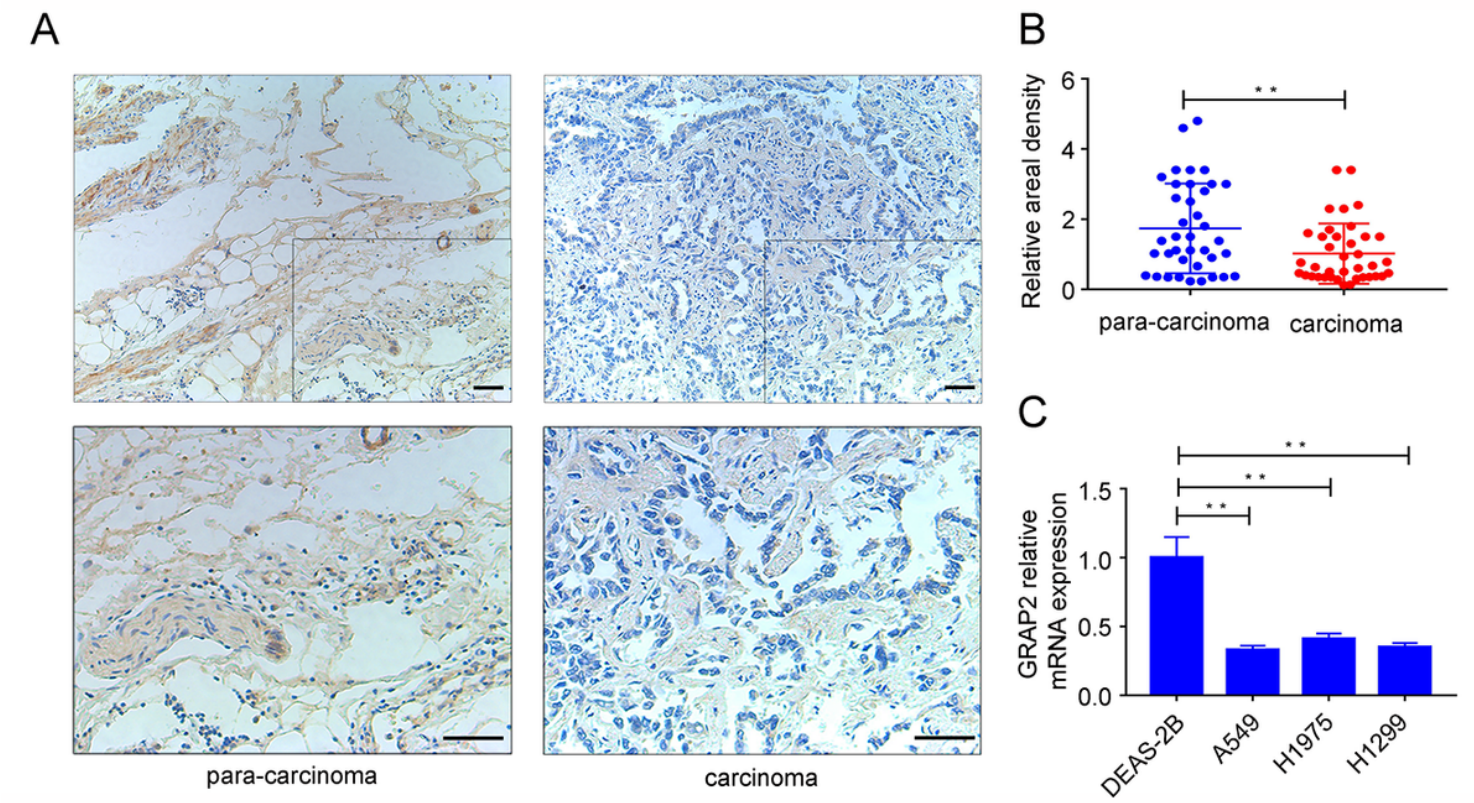

Figure 2

Protein expression of GRAP2 in lung cancer patients. (A) Immunohistochemical staining of GRAP2 was performed in tumor tissues and adjacent normal tissues. Representative images are shown. Scare bars, 50 μm. (B) The staining was quantified, as shown. The dot plot depicts the means and standard deviation of 76 images of tumor tissues and adjacent normal tissues. (C) GRAP2 expression in four different cell lines was examined by real-time PCR.

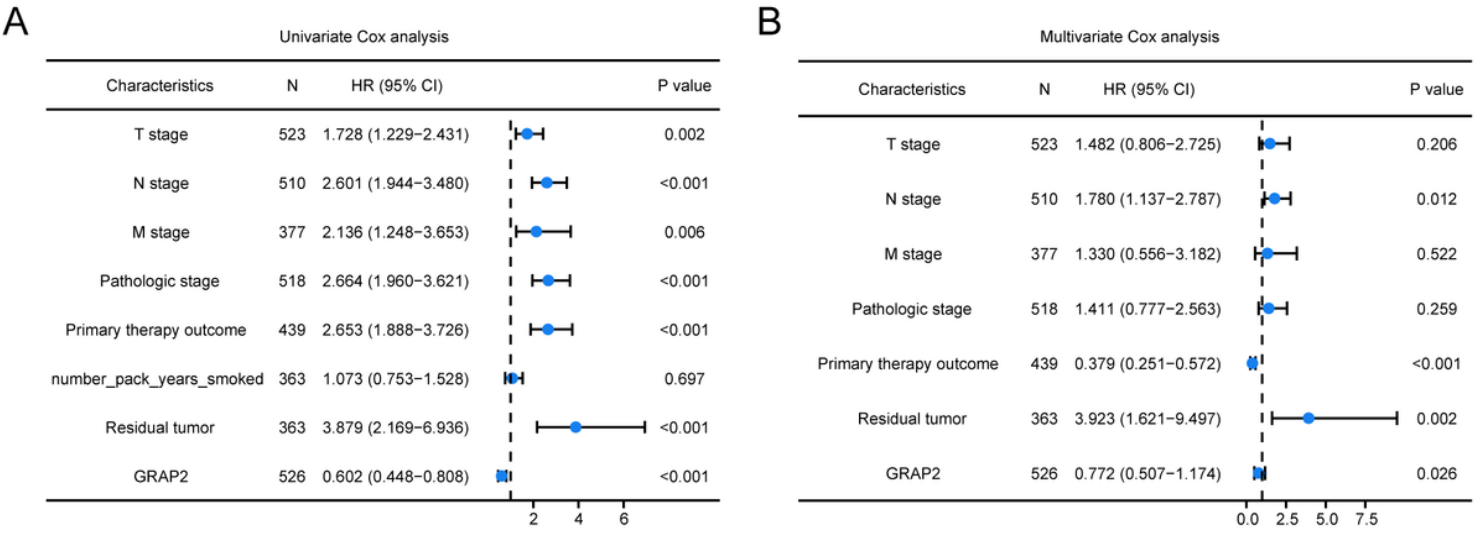

Figure 3

Kaplan-Meier survival curve analysis of the prognostic significance of high and low expression 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.

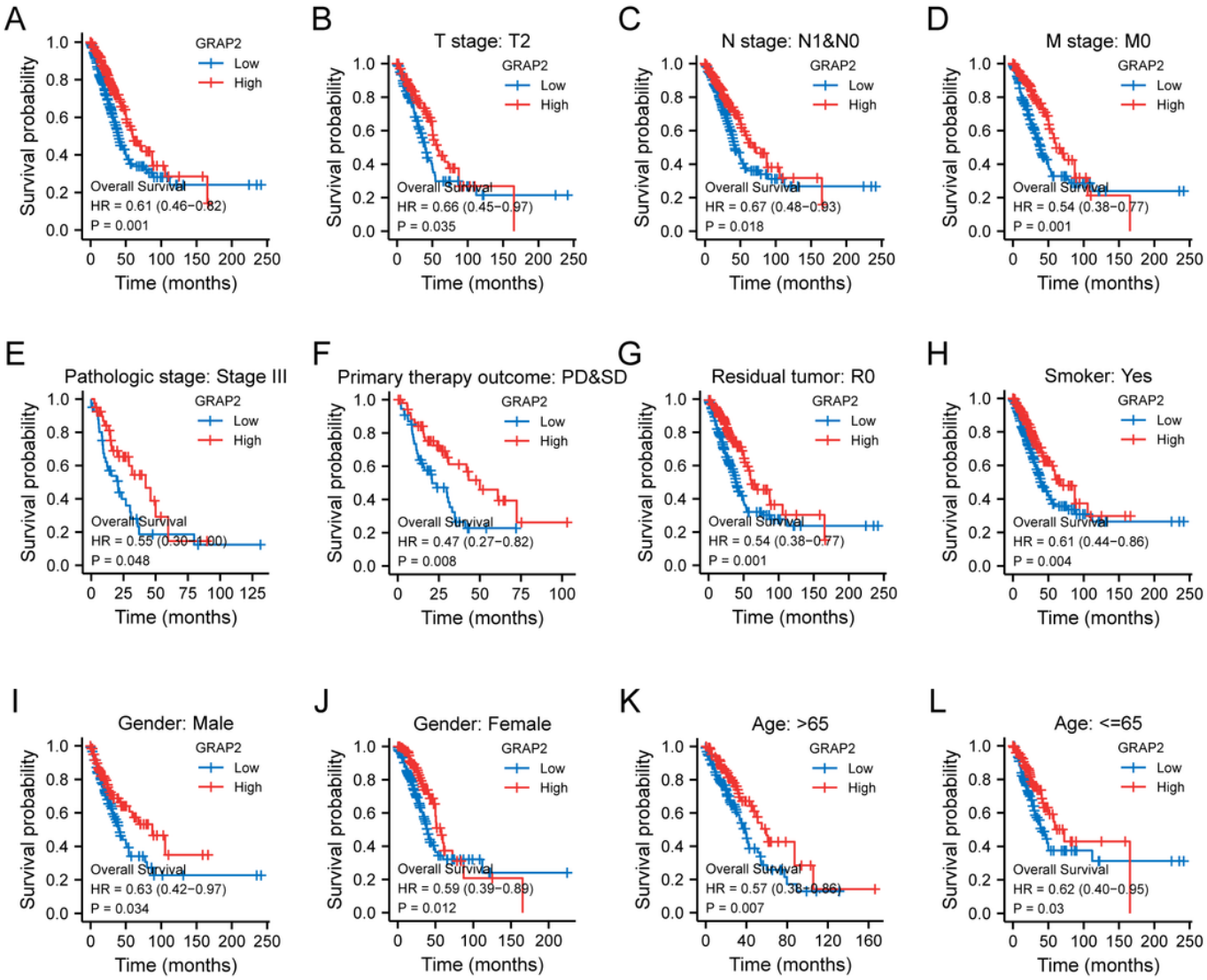

Figure 4

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 factors in the TCGA data set.

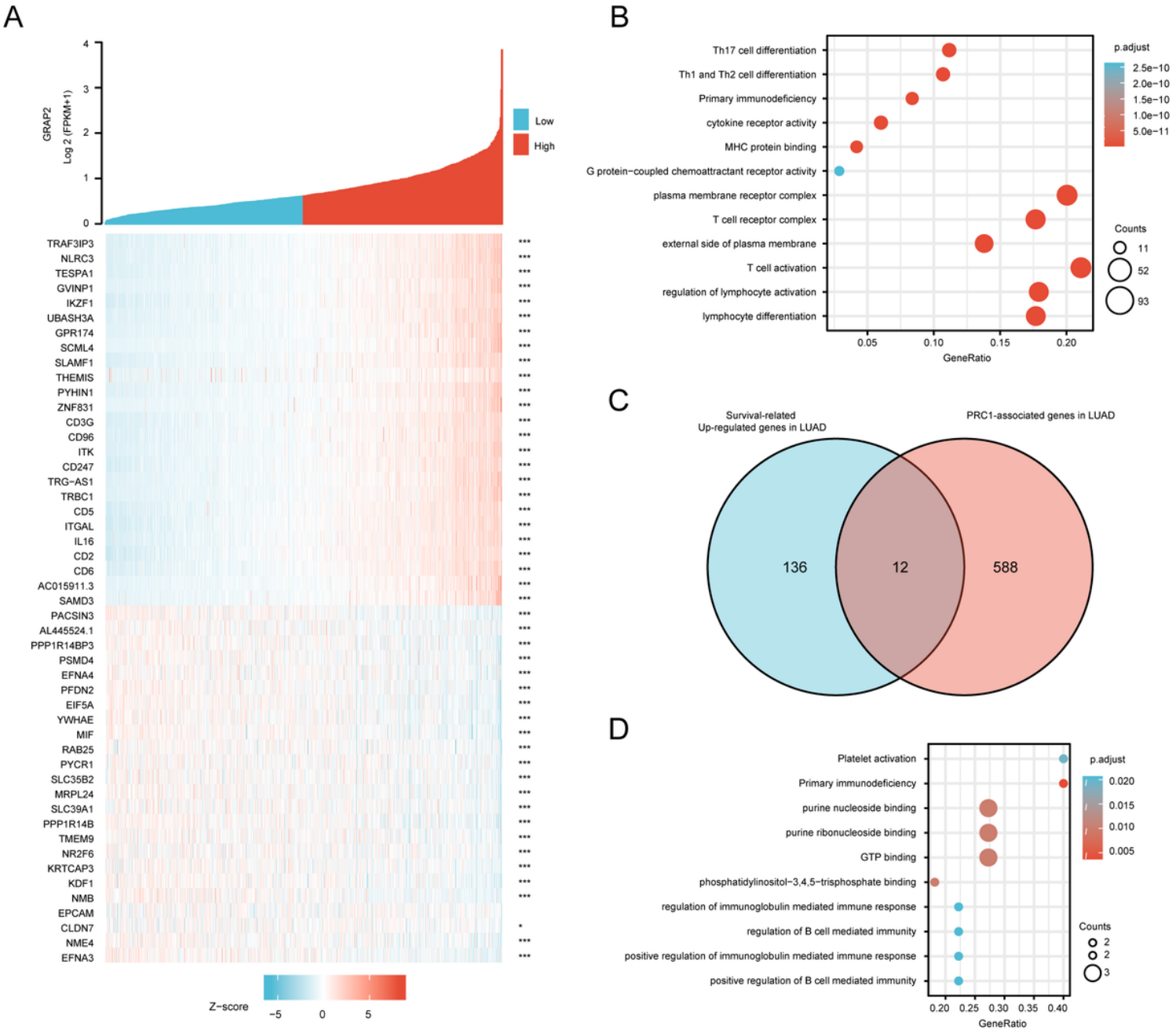

Figure 5

GRAP2 functional clustering and interaction network analysis of co-expressed genes of GRAP2. (A) The heat map shows the top 50 genes in LUAD that are positively and 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.

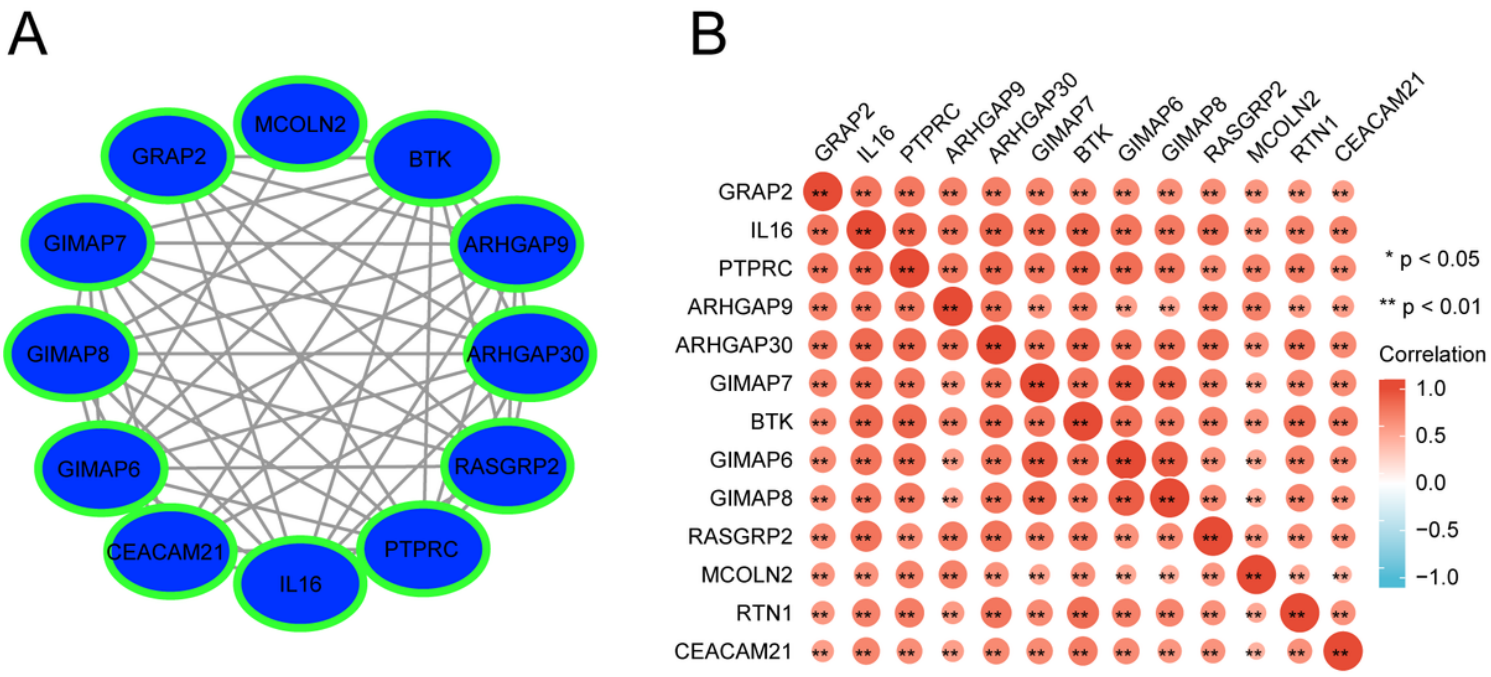

Figure 6

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

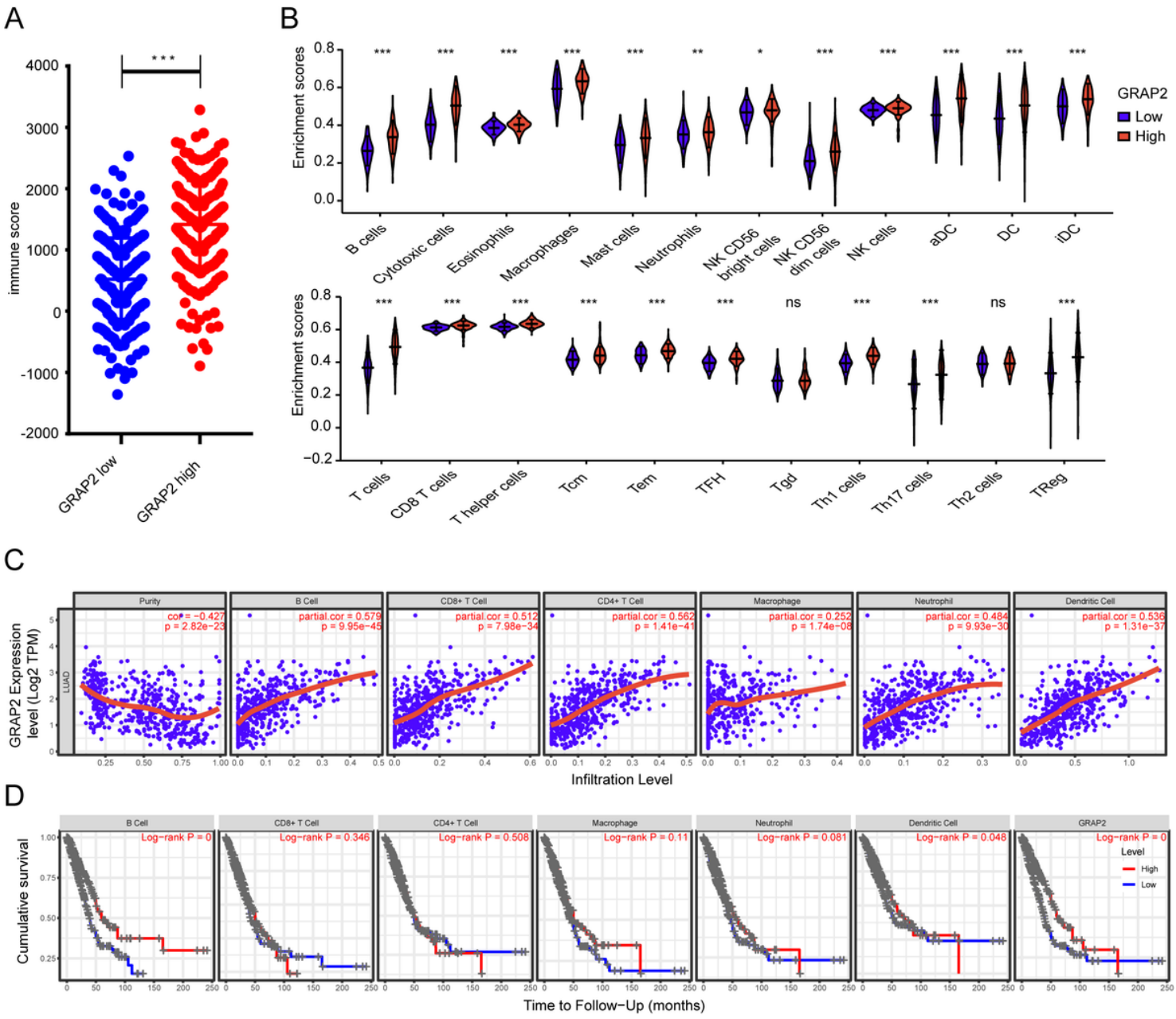

Figure 7

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 of GRAP2 high expression and low expression. The correlation between GRAP2 expression level (C) and immune infiltration and cumulative survival time (D) in B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells.

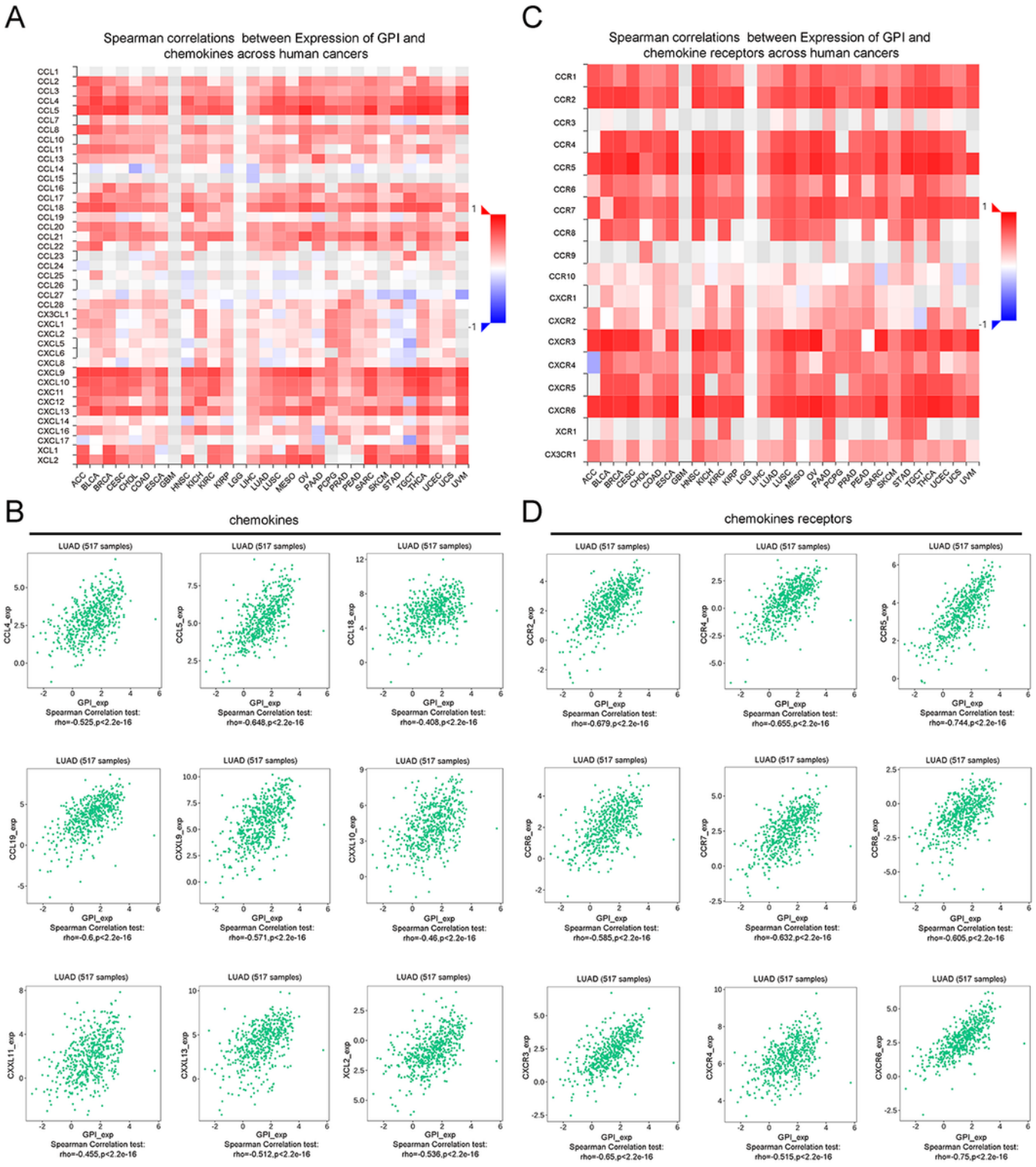

Figure 8

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 of the correlation between GRAP2 expression and chemokines /chemokine receptors in LUAD.

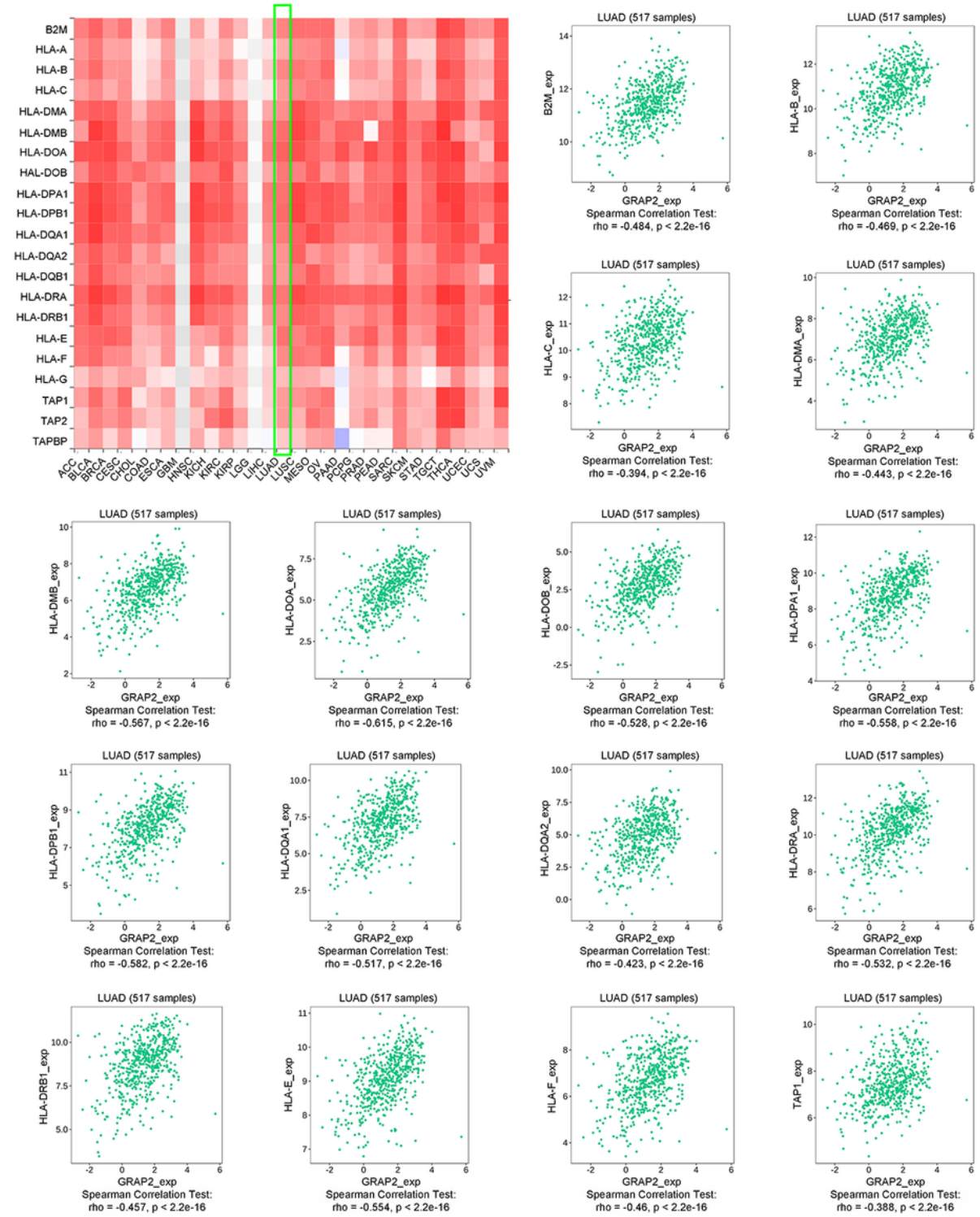

Figure 9

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